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LABORATORNAYA DIAGNOSTIKA BOLEZNEI RYB

G. D. Goncharov

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LABORATORY DIAGNOSIS OF FISH DISEASES

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120 pages with illustrations.

In this book are included the basic readily available methods for virological and bacteriological investigations of fish diseases, that are employed in medicine and veterinary science. The author recommends some of these on the basis of his own experience in ichthyology. The recommended methods will help ichthyologists to orient themselves in the routine work and to find the correct ways for studying diseased fish.

Particular attention is paid to collecting methods and methods of preserving native and fixed pathological material.

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AUTHOR'S FOREWORD

In the literature there is an absence of special methodological textbooks, devoted to the objective analysis of the causes of infectious diseases of fishes, which impedes to an extreme degree the everyday work of veterinarians, fish culturists and scientific workers in laboratories studying the diseases of fishes. In connection with this and on the basis of the many years of personal experience and the experience of our teachers (Professors M. A. Peshkov, G. B. Epshtein and E. I. Turevich), we draw attention to the organization of preparatory laboratory work, to the methods of collecting pathological material for bacteriological and virological investigations and also to the methods of the subsequent treatment of the original pathological materials for bacteriological, virological, histological and serological studies. 3

For the elucidation of the infectious nature of the pathogenic agent or for a demonstration of the pathogenic properties of bacteria isolated from pathological material, we recommend various schemes for the experimental infection of fishes in aquarium conditions or in natural conditions, while for diagnostic purposes we recommend serological methods of investigation, since the presence of the antibody-formation function in fishes has recently been acknowledged by all.

We present methods for preserving native and fixed pathological material for repeated investigations by means of

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* The figures in the right-hand margin indicate the corresponding pages in the original text.

the testing of filtrates on healthy fish, the demonstration of characteristic intracellular inclusions after the histological treatment of infected tissues, and also by means of the demonstration of a cytopathological effect on cell tissue cultures. In connection with the latter, attention is drawn to methods for obtaining cell tissue cultures of poikilothermic vertebrates, which have been elaborated here and abroad in recent years.

In the writing of this book the author made use of well known methodological textbooks in veterinary science and medicine and the works of Professor M. A. Peskov. Academician M. P. Chumakov and M. K. Voroshilova provided the opportunity for gaining a closer acquaintance with contemporary methods of tissue culture; Professor A. P. Zotov and Candidates of Biological Sciences A. I. Kanaev and N. I. Rudikov assisted in many ways with their advice. To all of these I express my profound gratitude.

Doctor of Biological Sciences
G. D. Goncharov

INTRODUCTION

The etiology, pathogenesis and diagnosis of infectious diseases of fishes have been extremely poorly studied. The data in the literature on these questions are not infrequently based on materials from casual studies and do not always correspond to the level of contemporary microbiology. All of this leads to a situation in which the role of the true pathogenic agent is often ascribed to a microbe which was isolated by accident from a diseased fish. As a result of an incorrect determination of the etiology there are often recommended unsuitable anti-epizootic measures.

In the contemporary information on the etiology of the infectious diseases of fishes there is still much that is unclear, and therefore at the present time there cannot be proposed any reliable method for the diagnosis of this or that disease.

Practically every time when we encounter a disease of a non-invasive character and one that is not caused by traumas of a chemical or mechanical nature, we find ourselves dealing with something new and unknown, since it is difficult to determine the disease by the external clinical features. This applies particularly to the dropsy-like diseases.

To obtain a deeper knowledge of the etiology of the infectious diseases of fishes, to study the properties of their causative organisms and also the intimate interrelationships between the micro- and macro-organisms it is necessary to unify the methodology of the virological and bacteriological investigations, in order that this be at the level of

contemporary scientific concepts. For this purpose use should be made of the rich experience of medical and veterinary virology and bacteriology, taking into consideration the specific reactivity of the organism. The latter is registered on the basis of the reciprocal reactions of the humoral and cellular defense systems of the organism, by means of which the pathogenic agent is also identified. 6

It is possible that in fishes, as lower vertebrates, the specificity of the defensive apparatus of the organism is expressed less distinctly than in man and the warm-blooded animals, and that it will be necessary in the future to elaborate more precise methods of differentiating specific features. At the present time however, in the first stages of the development of ichthyopathology, the adoption of existing methods of microbiology, as our practical experience has shown, brings definite benefits to the study of infectious diseases of fishes. In particular, serological methods may be recommended for diagnostic purposes in investigations on infectious diseases of fishes (G. D. Goncharov, 1962, 1966; V. P. Mikryakov, 1970).

METHODS OF BACTERIOLOGICAL INVESTIGATION

Bacteria are unicellular microorganisms. They do not contain chlorophyll and they reproduce by simple division (fission), and therefore they are referred to the class Schizomycetes (fission fungi). Morphologically, three basic forms of these are distinguished: spherical (cocci), rod-shaped (bacilli) and spiral (vibrions and spirilla).

Bacteria are visible through the normal light microscope. After special fixation and staining there can easily be distinguished in these, with high magnification under the microscope (immersion objective): the cell wall, the cytoplasmic membrane (periplast), the cytoplasm, the nucleus (nucleoid) and various inclusions (carbohydrate, lipoid, protein and others). Some species of bacteria have protective adaptations: a capsule (of a gel-like consistency), composed of various substances, and spores of round or oval form within the cell. Motile and non-motile bacteria are differentiated. Motility is dependent on the flagellar apparatus, composed of very fine protoplasmic filaments. In living bacteria these filaments are visible under the "dark-field" microscope, while in specimens killed by special fixation they may be seen through a normal light microscope after staining with a mordant.

Various species of bacteria are characterized by a particular set of enzymes, which determines their physiological characteristics.

The systematics of bacteria is based on their morphological and physiological characteristics. The identification of bacteria to species with the aid of the available textbooks presents certain difficulties. We recommend the use of the identification manual of Bergey (1957) for determining the species of various representatives of the bacterial flora of water and fishes.

Among all of the representatives of the bacterial flora of fishes, which are primary independent causative agents of fish diseases, or secondary agents which complicate the disease, there are known only aerobes and facultative anaerobes.

With respect to their culturing properties the species of the bacterial flora of fishes are similar to the species of bacteria of the intestinal group of man and warm-blooded animals. This permitted M. A. Peshkov (1939) to apply the scheme of investigation for the coli-typhi group of bacteria to the study of the causative agents of fish diseases and associated bacteria. The identification of the isolated bacterial cultures according to the scheme which is accepted for the study of the coli-typhi group of bacteria requires the usual bacteriological artificial nutrient media, the preparation of which is discussed below.

Preparation and sterilization of bacteriological vessels.

Glass vessels (test tubes, flasks, Petri dishes, graduated, Pasteur and Roux pipettes, and others) should be chemically clean and sterile. New glass vessels are immersed for an hour in a 25% solution of sulphuric acid, then rinsed in tap water and left in this until the end of the working day.

On the following day the vessels are again rinsed in tap water, flooded with distilled water and placed in an autoclave at a pressure of 0.5 at. for 20-30 minutes. Vessels that have previously been used are first decontaminated of the material that had been contained in them and washed with a small brush in a hot solution of mustard* (100gm per bucket of water), rinsed in tap water and then in distilled water. The washed vessels are placed in a drying chamber in such a way that all of the water would run off during the drying.

The openings of the test tubes and flasks are stoppered with dense cotton-wool plugs. The pipettes are stopped up with cotton wool at one end in such a way that when they are blown out the plug does not move down through the pipette, but also not too tightly. The test tubes are tied together in bundles (of ten). Each Petri dish and each graduated pipette is separately wrapped in paper. The Pasteur and Roux pipettes are placed into metal cases or wrapped in paper.

The bacteriological vessels are sterilized with dry heat in the drying chamber at a temperature of 160-170° for 1 - 1.5 hours or in an autoclave with saturated steam at a pressure of 1 at. for 15 minutes at a temperature of 120°.

Nutrient media which do not withstand high temperatures are sterilized in flowing steam in a Koch apparatus for 30 minutes or one hour every day or 3 times a day. Nutrient media which withstand a temperature of 112-115° are sterilized with saturated steam in an autoclave at a pressure of 0.5 - 0.6 at. for 20 minutes.

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*Translator's note. "gorchitsa" - mustard, Sinapis.

Preparation of basic nutrient media

Meat-peptone bouillon* **(MPB).** Fat-free beef is minced in a meat grinder. 1 kg of the ground meat obtained is covered with double that amount of distilled water and left at room temperature for 18-20 hours for extraction. In the summertime 60-80 ml of chloroform are added during the extraction, in order to retard the growth of bacteria.

The ground meat is squeezed out through 2 - 3 layers of cheese cloth and thrown away. The liquid obtained (meat juice) is boiled and filtered through filter paper, under which a thin layer of cotton wool has been placed. The meat juice, which has an acidic pH, may be preserved by adding chloroform to it. For this, 50 ml of chloroform is added to 1 liter of the meat juice in a chemically clean vessel, which is then tightly stoppered and shaken. It is stored in a cool dark place.

The meat juice is also prepared from fish meat, preferably zander or pike, since the meat of these has less fat. A more turbid extract is obtained from fish meat. For this 1 kg of fish meat is freed of the larger bones of the vertebral column, covered with two liters of distilled water and boiled for 3 - 5 minutes. Then this is cooled, the juice is carefully decanted, without stirring up the sediment and without squeezing out the meat.

For the standardization of the artificial nutrient media, the meat is flooded with distilled water, since the mineral composition of tap water varies.

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* Translator's note. "bul'on" is translated here by bouillon rather than broth, since the author subsequently apparently differentiates these terms, v. p. 15

MPB contains 1000 ml of the meat juice, 10 gm of dry peptone and 5 gm of sodium chloride.

The mixture of meat juice with peptone and salt is weighed in an enamel pan and is then boiled until the peptone is dissolved. After the boiling, distilled water is added to the MPB up to the initial weight. when the bouillon has cooled its pH is determined colorimetrically or with an LGU-01 potentiometer.

For establishing the required pH with the aid of the potentiometer, the electrodes are immersed in the vessel with the medium and a 1 N solution of sodium hydroxide is gradually added, stirring on an electromagnetic stirrer. With the colorimetric determination of the pH the amount of NaOH solution, required to neutralize the available volume of medium is calculated. After the addition of the 1 N solution of NaOH the MPB is boiled for 20 - 30 minutes. Its weight or volume is first established, so that, after the boiling, distilled water is added in an amount equal to that of the liquid that is boiled away. After cooling, the obtained bouillon is filtered through filter paper with a thin layer of cotton wool. The filtrate should be absolutely transparent. The filtered MPB is poured out into the test tubes (5 ml in each), flasks or large bottles for storage. The MPB is sterilized in an autoclave at a pressure which exceeds the normal air pressure by 1 at., at a temperature of 120° for 15 minutes. Generally after sterilization the pH decreases by 0.2.

The MPB for storage is preserved, like the meat juice, without autoclaving in a chemically clean vessel with the addition of chloroform in the amount of 30-40 ml per 1000 ml.

Meat-peptone agar (MPA). Finely cut-up agar (the best sort is Far-Eastern agar) is added to MPB which has a pH of 7.6. 20 gm of agar are taken for 1000 ml of MPB. When the agar swells in the bouillon, the liquid is boiled until it dissolves completely. As the agar is being dissolved the bouillon should be vigorously stirred. After the boiling, distilled water is added to the initial weight or volume. The melted agar in an enamel pan, covered with a lid, is placed in an autoclave and the pressure is brought up to 1 at. The heating is then discontinued and the pan is left until the following day, without opening the cover of the autoclave, so that the agar cools slowly. On the following day the gelled agar is separated from the walls of the pan and removed, and the settled sediment is cut off and thrown away. The MPA, which is cleaned in this manner, is cut up into small pieces and, after being weighed, is again melted in a pan. Distilled water is added to the melted agar to the initial weight and it is poured out in 5 ml portions into test tubes (for agar "slants") and in 10 ml portions (for future use in Petri dishes). The decanted MPA in the test tubes or other vessels is autoclaved at 1 at. for 15 minutes at a temperature of 120°. 11

A dry MPA preparation, which is convenient to use, is commercially available: the "dry nutrient agar" of the TsIEM.*

Meat-peptone gelatin (MPG). In alkaline MPB (pH 8.0) that is brought up to the boil there is gradually dissolved some nutrient gelatin. 15 - 20 gm of the nutrient gelatin

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* Translator's note. TsIEM - Tsentral'nyi Institut Epidemiologii i Mikrobiologii (Central Institute of Epidemiology and Microbiology).

(more in the summer) is taken per 100 ml of bouillon. After the gelatin is dissolved the liquid is clarified by coagulation. For this, the whites of two eggs are shaken up and added to the MPG, cooled to 30-35°, this is then vigorously shaken and placed in the flowing-steam Koch apparatus for an hour. During this time the the egg whites coagulate and remove all of the suspended matter.

The gelatin is filtered in the same Koch apparatus through a funnel with a dense layer of cotton wool. The MPG should be completely transparent. It is poured out into the bacteriological test tubes (in 3-5 ml aliquots) and fractionally sterilized in the Koch apparatus 3 times for one hour a day. As a result of autoclaving the MPG may lose its capacity of changing into the gel state. Gel-formation depends not only on the temperature but also on the pH of the medium (the pH should be alkaline).

Klodnitskii medium. To hot MPB (pH 7.6) is added glucose and melted MPA according to the following prescription:

meat-peptone bouillon (pH 7.6)	900 ml
glucose	20 gm
meat-peptone agar (1%) (pH 7.6) . .	50 or 100 ml (depending on the type of agar)

The melted MPA is added to the boiling MPB to obtain such a degree of viscosity of the medium that in a sample of the mixture (cooled) bubbles of air, formed by shaking the test tube, would rise slowly to the surface.

The prepared medium is decanted in 8 ml aliquots into bacteriological test tubes and autoclaved at 0.5 at. for 20 minutes at a temperature of 115°.

Peshkov's modification of Klodnitskii medium. To the Klodnitskii medium is added an indicator: a 1% filtered aqueous solution of neutral red in an amount of 3 ml per 1 liter of medium, or a 1.6% alcoholic solution of bromothymol blue to give the medium a blue colour. The medium is sterilized in an autoclave at a pressure of 0.5 at. for 20 minutes. 12

Solid Giss* media with carbohydrates and Andrade indicator. For studying the enzymatic properties of bacterial cultures, which digest carbohydrates, solid or liquid Giss media are utilized. The liquid Giss medium requires floats for catching the gas and therefore the solid Giss medium (with agar) is more convenient. For the identification of bacterial cultures 5 carbohydrates are most frequently utilized: glucose, lactose, mannitol, saccharose and maltose. To the basic Giss medium is added 0.5% of the carbohydrates. The composition of the basic Giss medium is:

dry peptone	20 gm
sodium chloride	5 gm
agar	5 gm
Andrade indicator	10 ml
distilled water	1000 ml

The peptone and salt are dissolved in boiling water. In the cooled mixture a pH of 7.4 is established, then the finely cut up agar is added and the mixture is weighed. After

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* Translator's note. "Giss" is a direct transliteration, but this name might also be "Hiss" or a phonetic equivalent of either of these possibilities.

the agar swells the mixture is boiled until the agar dissolves, distilled water is added to the initial weight and the Andrade indicator is poured in. The hot basic Giss medium is poured out in 200 ml portions into flasks or bottles. Into the different flasks is weighed out 1 gm of each carbohydrate and the corresponding inscription (glucose, lactose, mannitol, saccharose, maltose) is made on the paper cap of each flask. The well mixed carbohydrates are dissolved in the flowing-steam Koch apparatus and the media are then decanted into test tubes, an equal number being taken for each carbohydrate. On each bundle of test tubes should be written the name of the carbohydrate.

The Giss media which have been decanted into the test tubes are sterilized at a low pressure (0.5 at.) for 20 minutes.

Some sorts of peptone are contaminated with admixtures of carbohydrates. In the preparation of the Giss media it is very important to remove these. For this purpose there are added to the basic medium (without agar) yeast or bacterial cultures, which ferment all of the above mentioned carbohydrates, at the optimal temperature for the given culture. The presence of carbohydrate admixtures is revealed by acidification and gas-formation in the medium after inoculation of the culture. The prepared medium is sterilized in the Koch apparatus for 30 minutes, allowed to cool and then its pH is brought to 7.4. The subsequent procedure is as described above.

The Giss medium with the Andrade indicator should be colourless in the test tubes, while on acidification it becomes red. The medium is stored in a cool dark place. The test

tubes are appropriately labelled (with the name of the carbohydrate taken).

The composition of Andrade indicator is:

acid fuchsin	1 gm
distilled water	200 ml
sodium hydroxide (1 N solution)	32 ml.

Carbohydrate media for differential-diagnostic purposes with various indicators in the dry form (in a powder) are commercially available; these are dissolved in distilled water.

Minkevich medium. Fresh natural cow's milk is boiled, diluted twofold with distilled water and to this is added litmus tincture in the following proportions:

natural milk	500 ml
distilled water	500 ml
litmus tincture	30 ml.

For extracting the fat, 50 ml of chloroform is added and the mixture is vigorously shaken several times, the bottle having been plugged with a rubber stopper. On the following day, the fat which has been dissolved in the chloroform will subside to the bottom. The fat-free milk is decanted or siphoned off, and is autoclaved at 0.5 at. for 20 minutes. Litmus milk prepared for storage is preserved without autoclaving with chloroform in a dark cool place. Clarified litmus milk with signs of autolysis is unsuitable.

The litmus tincture is prepared in the following manner: 10 gm of dry granulated litmus is ground up in a porcelain mortar to a powder-like state, it is transferred into

a flask and flooded with 100 ml of 96% ethyl alcohol. This is placed in a thermostat for 3 days at a temperature of 37°, and during this time the coloured alcohol is decanted daily, without shaking up the deposit, and the decanted alcohol is replaced by the same amount of fresh alcohol. After the extraction the deposit is filtered and dried on filter paper in the thermostat. The dried residue is dissolved in 100 ml of distilled water for three days in a thermostat at a temperature 14
of 30°. The tincture is decanted from the colourless residue and conserved with chloroform (to 50 ml of the tincture is added 3-4 ml of chloroform). It is stored in a dark cool place.

Meat broth according to Khottinger* The basic solution of Khottinger broth is prepared in the following manner: 1 kg of meat, cut up into small pieces, is placed into 1.5 liters of boiling distilled water for 20 minutes. Then the meat is removed and minced in a meat grinder. The meat stock is decanted into a 2-liter flask or bottle, into which the ground-up meat is also added. To this is added 1.5 gm of soda, 3-5 gm of pancreatin (at a temperature of the meat stock no higher than 45°) and 20 ml of chloroform. The flasks with the mixture are vigorously shaken, tightly closed with a stopper and placed in a thermostat for 4 days at 37° for digestion. After the completion of the fermentation, the meat is deposited on the bottom of the flask in the form of a fine-grained mass; the liquid above the deposit should be transparent. The prepared solution gives a positive reaction for tryptophane and peptone.

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* Translator's note. Direct transliteration: Possibly Hottinger.

For obtaining a qualitative reaction for tryptophane 2-3 ml of the broth are slightly acidified with 5% acetic acid, bromine water is added drop by drop until the appearance of a pink-violet colour in the presence of tryptophane.

For obtaining a qualitative biuret reaction for peptone 1 ml of the broth is diluted with 4 ml of distilled water, to the diluted broth are added 2 ml of a 33% solution of alkali (NaOH or KOH). After shaking, there are carefully added along the wall of the test tube a few drops of a 1% solution of copper sulphate. In the presence of well formed peptones in the broth, at the border of the two layers of liquid there appears a red ring. Whole proteins give a dark-blue to violet ring, albumoses - a violet ring.

Reagents for determination of tryptophane: 2-3 ml of bromine water, and 100 ml of distilled water. Reagents for the biuret reaction: 33 gm of caustic soda (NaOH) or caustic potash (KOH) and 100 ml of distilled water; 1 gm of copper sulphate ($CuSO_4$) and 100 ml of distilled water. If during the course of the first two days the fermentation of the meat in the thermostat is arrested, then the broth is alkalized to a pH of 7.8 - 8.0; the effect of the pancreatin is then intensified.

Khottinger bouillon. The basic solution of Khottinger broth is diluted five-fold with a salt solution of the following composition:

distilled water	1000 ml
sodium chloride	7 gm
dibasic potassium phosphate (K_2HPO_4) . . .	1 gm
tribasic calcium phosphate (saturated solution) ($Ca_3(PO_4)_2$)	5 drops

The diluted solution is filtered and poured out into test tubes. These are then sterilized by autoclaving for 15 minutes at a pressure of 1 at.

Koser citrate medium. The composition of this is as follows:

sodium-ammonium phosphate ($Na(NH_4)_2PO_4$) . .	1.5 gm
monobasic potassium phosphate (KH_2PO_4) . .	1.0 gm
magnesium sulphate ($MgSO_4$)	0.2 gm
sodium citrate	3.0 gm
distilled water	1000 ml

This is poured out into test tubes in 5 ml portions. These are sterilized by autoclaving for 20 minutes at a pressure of 0.5 at. The citrate test is diagnosed by the formation of a cloudiness in the medium.

Shmits-Shandel'*medium as modified by Peshkov. The composition of this is as follows:

meat-peptone bouillon (fermented)	1000 ml
agar, far-eastern (or imported)	20 gm
Congo red (3% aqueous solution)	100 ml
saccharose (30% aqueous solution)	50 ml
NaOH (4% solution)	5 ml

Method of preparation of medium. Sterile MPB (pH 7.6), cooled to 37°, is inoculated with an 18-hour culture of coliform bacteria (E. coli) for fermenting the carbohydrates. After 24 hours of fermentation the medium is sterilized in the Koch apparatus for 30 minutes, cooled and the pH regulated to 7.6.

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* Translator's note. This is a direct transliteration; could be Smith (or Schmidt)-Chandelle or some phonetic equivalent of this. (Schmitz - ?).

Finely cut up agar is added, this is allowed to swell up over the course of 2 hours; the mixture is then weighed and boiled, with vigorous stirring until the agar is dissolved. To the MPA distilled water is then added to the initial weight. The mixture is then placed in the autoclave to clarify the agar (as in the preparation of the normal MPA). The transparent agar is decanted into bottles in 100 ml portions and sterilized for 15 minutes at a pressure of 1 at.

The 3% aqueous solution of Congo red is poured out into test tubes in 10 ml portions and autoclaved.

The 30% aqueous solution of saccharose is poured out into test tubes in precise 5 ml aliquots and these are sterilized for 20 minutes at a pressure of 0.5 at.

The 4% alkali solution (chemically pure NaOH) is prepared in a sterile flask with sterile distilled water.

The medium is prepared aseptically in the following order. The MPA is melted. To 10 ml of the sterile Congo red solution are added, using sterile pipettes, 0.5 ml of the alkali solution and 5 ml of the sterile saccharose solution. The mixture is warmed on a water bath to 80°. Then it is poured into the melted MPA, this is well mixed by stirring and poured out into the Petri dishes. The medium should be transparent and have a ruby-red colour.

Endo medium with lactose (or saccharose for <u>Aeromonas punctata</u>). The composition of this is as follows:

meat-peptone bouillon (fermented)	1000 ml
agar, far-eastern	20 gm
basic fuchsin (saturated alcoholic solution)	5 ml

sodium sulphite (10% aqueous solution) . . 25 ml
lactose (50% aqueous solution or
saccharose) . . . . . 20 ml

Method of preparation of the medium. The previously prepared MPA, on fermented bouillon (see preparation of the Shmits-Shandel' medium), is melted in volumes of 100, 200 and 500 ml and to this are added in a sterile manner, calculated per 1 liter, the above mentioned ingredients in the following order:

20 ml of a 50% solution of lactose or saccharose in distilled water, sterilized in an autoclave for 20 minutes at a pressure of 0.5 at;

a mixture, which has been boiled for 10 minutes, of 5 ml of a saturated alcoholic solution of basic fuchsin and 25 ml of a 10% aqueous solution of sodium sulphite with sterile distilled water. The mixture should be of a pale-pink colour and transparent. The colour of the mixture depends on the batch of fuchsin and the quality of the sodium sulphite, and therefore a greater or lesser quantity of the latter is 17
sometimes added. The sodium sulphite solution should be freshly prepared each time. After a thorough mixing of the ingredients, avoiding foaming, the medium is poured out into Petri dishes.

Clarke medium. This is employed for checking on the formation of acetyl-methyl-carbinol (Voges-Proskauer reaction, V.P. test) and a change in the pH of the medium using methyl red (M.R. test). The composition of Clarke medium is:

dry peptone	5 gm
glucose	5 gm
dibasic potassium phosphate	5 gm
distilled water	1000 ml

Method of preparation of the medium. The peptone, glucose and potassium phosphate are ground up in a mortar and dissolved in 800 ml of distilled water, while being heated in a water bath and stirred for no less than 20 minutes. After dissolution, this is filtered through filter paper, allowed to cool to 20° and distilled water is added to 1 liter. The medium is sterilized only in the flowing-steam Koch apparatus for one hour three times in a day. The medium should be completely transparent and without a deposit. When chloroform is added to it, it may be stored without sterilization.

Medium for denitrifying bacteria. For the determination of the denitrifying capacity of the bacteria, to 1000 ml of normal MPB (pH 7.4) are added 5 gm of potassium nitrate (KNO_3). Prior to this the absence of nitrites is checked in the bouillon and saltpetre (separately), utilizing the reagents which are mentioned below (in the section on "Reduction of nitrites"). The medium is sterilized in an autoclave for 15 minutes at 1 at.

Starch agar. To the MPA is added 0.5% soluble starch: the agar is melted and the dissolved starch is poured in. The mixture is thoroughly mixed, while being warmed on a water bath, and poured out into test tubes in portions of 12 - 14 ml. These are sterilized in a Koch apparatus for one hour three times in a day.

Meat-peptone agar with fat. To MPA in a test tube (10 ml), intended for being poured out into Petri dishes, is added 0.5 ml of unsalted high quality butter. The test tubes with the butter are sterilized at 120° (a pressure of 1 at.) for 15 minutes in an autoclave. The melted agar with the butter is poured out into Petri dishes, after the mixture has been well emulsified.

Potato medium. From a large, cleaned and washed potato there are cut out cylindrical pieces with a cork borer, these pieces are cut in two diagonally and placed into a 1% aqueous solution of sodium bicarbonate for 2 hours. Each piece is placed separately into a test tube, in the bottom of which is placed a glass tube in a small amount of tap water. Such an arrangement of the piece of potato prevents it from being boiled to a pulp during sterilization and from drying out subsequently.

The potato is sterilized in an autoclave at a temperature of 115° (at a pressure of 0.5 at.) for 20 minutes.

Gorodkova medium. For establishing the capacity of bacteria for spore-formation a hard agar medium of the following composition is utilized:

peptone	1.0 gm
meat extract (paste)	0.5 gm
sodium chloride	0.5 gm
glucose	0.25 gm
agar-agar	1.0 gm
distilled water	100 ml

The mixture is boiled until the agar dissolves, poured out into test tubes and sterilized in an autoclave at a pressure of 0.5 at. for 20 minutes.

Tarr medium. Spore formation in bacteria is verified on a "semi-starvation" medium of the following composition:

saccharose	0.1 gm
ammonium phosphate	0.1 gm
sodium chloride	0.1 gm
crystalline magnesium sulphate	0.05 gm
potassium dihydrogen phosphate (KH_2PO_4)	0.05 gm
ferric chloride	trace
agar-agar	2.0 gm
distilled water	100 ml

Method of preparation. The agar is allowed to swell in the medium, this is weighed and then boiled until the agar is dissolved; distilled water is added to the initial weight, the medium is poured out in 5 ml portions into test tubes and autoclaved for 15 minutes at a pressure of 1 at.

Sampling pathological material for bacteriological study

In the sampling of pathological material it is necessary to pay attention to the principles of asepsis. Only a live fish is taken for bacteriological study, since in fish saprophytes develop very rapidly after death, which makes it more difficult to determine the pathogenic agents.

Scrapings from the outer covering of the body or from the gills of the fish are obtained for bacteriological

inoculation in the following manner, so as to avoid extraneous bacteria from being taken in the sample. If the diseased fish is being caught directly from a body of water, then the bucket is first flamed right on board the boat. For this, 15 - 20 ml of alcohol is poured into the bucket, its sides are rinsed with this and a lighted match is thrown into the bucket. Then with this same bucket some water is taken up from the body of water, the hands are washed with alcohol and a fish is picked for investigation directly from the net in such a way that it is not touched by the hands of the fishermen. Inoculations are made only in an enclosed room (or in a tent), protected from wind. The fish is removed from the bucket with hands that have been washed in alcohol.

In the work area in the enclosed room there should be prepared: a spirit lamp; a rack with test tubes of Klodnitskii medium; Petri dishes with MPA or medium with Congo red for the inoculation of blood; a bacteriological platinum loop; Pasteur and Roux pipettes; a pencil or special ink for glass; microscope slides in a jar of alcohol; a ground glass for preparing blood smears; methyl alcohol for the fixation of the blood smears and impressions (or Nikiforov fluid - a mixture of equal parts of ethyl alcohol and ether); strips of filter paper for absorbing blood when organs are sectioned; cotton wool; instruments: scissors, scalpels, spatulae, surgical and anatomical forceps, Péan clamps in a a jar with alcohol; a sterile gauze mask for protection against droplet infection (in a paper packet) and sterile gauze dressings.

The fish is held on the scales, by gripping the eyes of the fish with two fingers of the left hand (the right hand of the laboratory worker holds in readiness a test tube with medium). With the bacteriological platinum loop there is quickly made a scraping from the infected portion of the outer skin of the body of the fish and the material obtained is inoculated into the Klodnitskii medium (the technique of inoculation is described below). In a parallel manner a control 2
inoculation is made from an uninfected portion of the body of the fish.

If the diseased fish is brought in to the laboratory, after having been fortuitously found by fishermen, then, before sampling the pathological material, the external lesions are washed several times with sterile physiological saline or sterile water. If the lesions embrace the layers of subcutaneous and muscular tissue, then, after cauterization with a spatula, a deep penetration is made with a Pasteur pipette through the scab that is formed and by means of a rubber bulb, or by mouth, material is sucked up and inoculations are made in Klodnitskii medium but not in MPB. Aerobes and anaerobes develop well in Klodnitskii medium; antagonism is delayed in the development of mixed primary cultures. However for the storage and transportation of cultures the Klodnitskii medium should not contain glucose, since the products of fermentation of the latter have an adverse effect on the bacteria.

For the investigation of the blood, internal organs and intestinal content the fish is fastened with a gauze bandage to a board (Figure 1), which has been washed with a 5%

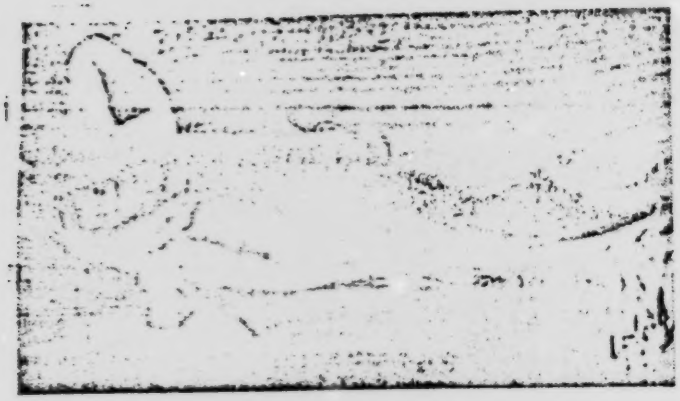

Рис. 1. Прикрепление рыбы к станку.

Figure 1. Attachment of fish to board.

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solution of lysol or with carbolic acid. First there is made an inoculation of the blood, then of the contents of the posterior section of the intestine and, finally, the abdominal cavity is cut open. The blood is obtained from the heart or the caudal vein. It is easy to take blood fron the heart of cyprinid fishes. To do this, the descaled skin between the pectoral fins is cauterized and through the scab that is 21
formed a puncture is made with a sterile Pasteur pipette. With a twisting movement the pipette is deeply inserted at an angle of approximately 60^{o} until the appearance of blood in its capillary. It is very important to watch out that the wall of the gill cavity is not pierced, since this would violate the sterility of the pipette. It should be borne in mind that in some bony fishes within the compact pericardial sac there is a transparent fluid; a particularly large amount of this is present when the fish is diseased. Blood from the heart may also be obtained by a puncture through the wall

which separates the gill cavity from the cardiac cavity. The first portion of blood is rapidly transferred into a Petri dish with a solid agar medium, the second - into a test tube with Klodnitskii medium, and the third - onto a microscope slide for smears. In the Petri dish 2-3 drops of blood are smeared over the surface of the agar with a Drigal'skii spatula; the test tubes with the blood are shaken up; on the microscope slides the the laboratory technician makes a thin smear from the small droplet. The smear is made rapidly so that the blood does not have time to coagulate. On the test tubes and Petri dishes is indicated the number of the sample; on the smears after the thin film of blood has dried the number is placed on a corner of the microscope slide. After drying the smears are fixed in methyl alcohol (preferably dehydrated) for 2-3 minutes or in alcohol-formalin (aa) for 20 minutes or in a mixture of the (aa) alcohol with ether. One smear is stained with Giemsa stain, another with the Gram stain.

The inoculation of blood onto the solid MPA medium in a Petri dish generally gives a distinct quantitative representation of the presence of bacteria in the blood stream of the fish in conjunction with the bacterioscopic data from the blood smears. Inoculations of blood and internal organs onto Shmits-Shandel'e medium or onto Endo medium (in the latter lactose is replaced by saccharose) permit one to rapidly isolate Aeromonas punctata, which was previously considered to be the causative agent of infectious dropsy but has now been acknowledged as a secondary pathogen accompanying the virus infection.

The remaining portion of the blood is used for obtaining serum, which is required for the immunity reactions. The serum is sucked up from a clot of coagulating blood by means of a Roux pipette, which is sealed at its point of constriction on the flame of a spirit lamp.

For obtaining blood from the heart of large bony and cartilaginous fishes the abdominal cavity is cut open. The fish is cleaned of scales and slime on its left side, the rays of the pectoral fin are cut off and this side is carefully rubbed with a cotton wool swab that has been wetted with alcohol or a 5% alcoholic solution of iodine, and, if a gas burner is available, the side is flamed. The operation is carried out in a gauze mask. A scalpel, moistened with alcohol, is passed through the flame of a burner and then is used to pierce the wall of the abdominal cavity a little above the anus, without touching the intestine. Into the cut is introduced the blunt end of a pair of scissors and carefully, without damaging the internal organs, the cut is elongated in a semicircle towards the lateral line and towards the head, and then down behind the base of the pectoral fin (Figure 2). The abdominal wall is turned back using a sterile Péan clamp and then all of the internal organs are carefully moved aside with sterile anatomical forceps (without damaging the intestine!), freeing the septum of the pericardial cavity. The heart is penetrated with the capillary of a Pasteur pipette through the connective septum which separates the pericardial and abdominal cavities or through the wall of the pericardium after cutting open the pericardial cavity.

Рис. 2. Вскрытие брюшной полости рыбы.

Figure 2. Dissection of abdominal cavity of fish.

The method of obtaining blood from the caudal vein is more complex. By severing the caudal peduncle much blood can be collected from the vein but this method is not suitable for bacteriological investigation. Material for inoculation from the internal organs is taken by Pasteur pipettes.

After the inoculations are made, one turns to the preparation of the klatsch-preparations (impressions) of the infected and non-infected sections of the skin and internal organs (see chapter on virology) for staining with the Giemsa and Gram stains.

In the collecting of the pathological material it is necessary to maintain very precise records and observe a very precise sequence, since there are generally accumulated many different bacteriological cultures and data on pathologico-anatomical and clinical changes. M. A. Peshkov has proposed a special index card for recording data on bacterial cultures (Appendix). This card allows one to take into account the

information necessary for diagnostic purposes. In addition, a log is kept of all of the investigations, with a single number assigned to any given object. Thus, the strains of bacterial cultures, sera, smears, impressions, excised pieces of tissue for pathological histology from every investigated object have a single serial number. In order to distinguish the differentiated strains of bacterial cultures, letters are appended to the numbers in alphabetical order, as is the site from which the bacterial sample was taken (skin, blood, intestine etc.). The results of the primary inoculations are recorded in the log, noting the growth or absence of growth of the bacteria in the storage medium.

Isolation of pure bacterial cultures

The term pure bacterial cultures refers to cultures which have been obtained from a single isolated cell. Separate bacterial cells are isolated by means of a micromanipulator, however in mass production bacteriological work a bacterial suspension of an agar or bouillon culture is generally attenuated (to a single cell)*, with the subsequent obtaining of a colony that has grown out of this cell. The suspension is attenuated either by by means of smears on the surface of nutritive agar in Petri dishes or by dilution of the bacterial mass or suspension in sterile tap water, subsequently inoculating the diluted suspension into one or two Petri dishes with agar.

Translator's note. (...) omitted in original text.

For isolating a pure culture the mixed agar culture is flooded with sterile tap water or physiological saline (but not with distilled water) and a bacterial suspension is obtained. Then melted MPA is poured out into Petri dishes and the gelled nutritive agar is divided into several sectors; by means of a platinum loop a drop of the bacterial suspension obtained is applied onto the surface of one sector. The drop of suspension is spread successively over the sectors with a Drigal'skii spatula using a circular movement over the surface of the agar in the first sector, then with the same spatula - in the second sector, etc. In this manner, into the last sector there are inoculated separate, well isolated bacterial cells, which grow into individual colonies as a result of their reproduction by division after 24 - 48 hours at a temperature of 26 - 30°.

During the first day the growing colonies are little differentiated from one another morphologically and therefore the pure cultures of the bacterial flora of fishes are singled out after 2 - 3 days, when the colonies attain a definite configuration and structure. Such colonies are sketched or photographed under low microscopic magnification (without the condenser), after which from these there are subcultured strains of pure cultures on MPA slopes in test tubes. Since the cells of various species of bacteria may be situated close to one another on the agar and may fuse into one colony during their development, the purification of the culture is repeated 2 - 3 times by this same method on Petri dishes.

Bacterial colonies of different configuration and structure are subcultured on agar slopes in test tubes and are placed in a thermostat at a temperature of 26 - 30°. The 24-hour agar cultures in the test tubes are examined under a magnifying glass (over a light). Homogeneity of growth of the culture in the test tube, which is evident under the magnifying glass, is indicative of its relative purity. Smears of the culture are stained with Gram stain and examined microscopically with an immersion objective. Thus, the purity of the bacterial culture is determined by the comparison of the homogeneity of the colonies in the Petri dish on agar (by the structure), of the homogeneity of growth of 2-3 day cultures in test tubes on agar slopes and of the homogeneity of the morphological and staining properties of the cells from a 24-hour agar culture in a smear stained with Gram stain.

The primary bacteriological material in the inoculations of the intestinal contents, in the inoculations from the mucus of the skin, gills etc. generally contains several species of bacteria. Therefore such a mixed initial culture is inoculated by means of the attenuating smears according to the method described above. If the mixed initial culture on MPB is kept at room temperature, then after 3 - 5 days, because of the antagonistic properties, one species of bacteria may displace the other species, and in the initial culture there will remain only one species of saprophyte, while the pathogenic species, which are less adapted to the artificial food medium, disappear. As our experience has shown, the application of Klodnitskii medium for the primary inoculations allows 2.

one to isolate a larger number of species of bacteria than does MPB. The elective and selective media with bile, for the coli-typhus group, have not, up to the present time, gained wide application for the bacteriological culturing of pathological material from fishes.

In laboratory conditions the primary inoculations are made by means of the attenuating smears directly on agar in Petri dishes or on the differential agar media with indicator and carbohydrates (Shmits-Shandel'e, Endo, Levina media and others), the fermentation of which is characteristic of particular species of bacteria.

The strains of bacterial cultures which have been checked as to their purity are subcultured on agar slopes for obtaining a "working" culture and on Klodnitskii medium (though without glucose) for the purpose of preserving a duplicate culture. The cultures are kept in a thermostat until the appearance of an abundant growth and are then placed in a refrigerator for storage at a temperature of 1 - 3°. The "working" culture on agar is reinoculated after 2 - 3 days, while the duplicate culture on the semi-liquid Klodnitskii agar without glucose is recultured no less frequently than once a month.

Cytological study of bacteria

Motility of bacteria. The study of living bacteria permits one to establish their capacity for locomotion, and also to determine the character and intensity of their motility.

The existing classical method of determining the motility of bacteria in a hanging drop in a moist chamber on a cover glass may be considerably simplified and expedited, as far as this is permitted by the bacterial flora of fishes which is non-pathogenic to man.

A drop of a 24-hour bouillon culture is placed onto a microscope slide with a platinum loop, the edge of the drop is found under low magnification of the microscope (X10 oc., X10 ob.), the condenser is lowered. Then, using a OI-7 (or OI-19) light source, one changes to a higher magnification (X10 oc., X40 ob.), raising up the condenser of the microscope until critical even illumination is obtained, at the same time using the edge of the drop to keep oneself oriented. When an object for study is found, the tube of the microscope is let down by means of the fine adjustment.

The independent motion of bacteria should be distin- 26
guished from Brownian movement. In Brownian movement, if there is no current flow of the liquid in a drying drop, the bacteria oscillate in various directions from one point, remaining in one place. Therefore, movement is observed in the center of the drop. The bacteria that are independently motile move either in a straight line or in a spinning and zig-zag fashion in one or a few directions. The character of the locomotion depends on the structure of the locomotory flagellar apparatus. On this basis it is sometimes possible to determine whether a bacillus is peritrichous or monotrichous (see below).

The rate of locomotion of bacteria is noted according to a 4-degree scale: very weak (+), weak (++), rapid (+++) and very rapid (++++).

M. A. Peshkov recommends that the motility of bacteria be determined in a drop of tap water at the cultivation temperature of the culture (26 - 30°), i.e. at the optimal temperature for growth of the bacteria, which should be established right at the beginning of the study of the cultures.

Staining of flagella. The best method for revealing the flagellar apparatus in bacteria is the method of Löffler as modified by M. A. Peshkov. With abrubt changes in temperature and mechanical jolts the flagella of bacteria easily fall off. Therefore the test tube rack with the cultures on agar slants and with the test tubes with tap water for preparing the fine suspension of bacteria are first placed in a thermostat without sudden movements. During the staining of the flagella it is necessary to observe the following conditions:

an 18-hour agar culture is carefully transferred with a cool loop or with a fused capillary of a Pasteur pipette into a test tube with tap water (5-6 ml) at the same temperature (no shaking!). The loop or capillary with the culture is carefully immersed in the water and left in it for no less than 10 - 15 minutes. The suspension obtained should be almost transparent;

the bacteria are fixed in a 1-2% solution of osmic acid for 1-2 minutes. For this, two equal drops, of the osmic acid and of the bacterial suspension, are placed side by side onto a watch glass with the aid of a fine capillary. The 2 equal drops of the fixative and culture are combined into one large drop, the mixture is covered with another watch glass which prevents this from becoming dessicated;

well degreased cover glasses, no more than 0.18 mm in thickness, are prepared. For this they are treated with potassium bichromate and then with concentrated nitric acid for 2-3 days, after which they are thoroughly washed in distilled water and then in 96% alcohol. They are also stored in 96% alcohol (in a jar); 27

a clean cover slip is taken from the alcohol with forceps (most conveniently with Korne forceps), passed through the flame of the burner and allowed to cool. From the fixed bacterial suspension on the watch glass, using a finely drawn out capillary of a Pasteur pipette, there is taken up a small amount of the liquid with the bacteria and 5 very small droplets are applied to the flamed surface of the cover glass (onto the slightly convex surface!), one droplet in the center and 4 in the corners. On a well degreased cover glass the drops spread out and and rapidly dry out;

the dried out drops on the cover glass are treated and stained according to the following process.

The mordant is composed of three components: a saturated alcoholic solution of basic fuchsin (to 1 gm of basic fuchsin in a test tube is added 10 ml of 90% ethyl alcohol and this is placed for 1-2 hours in a thermostat at 50°); a 20% aqueous solution of tannin (of good quality); a saturated, in the cold, aqueous solution of Mohr's salt (ferrous ammonium sulphate).

1 ml of the fuchsin solution is mixed with 10 ml of the tannin solution and to this mixture is added 5.5 ml of the solution of Mohr's salt. Better results in the mordant treatment are obtained when working with a mixture which has ripened

over the course of a week or more. The mordant should have an alkaline reaction, and therefore in cases of poor results to each 10 ml there is added 0.1 - 0.2 ml of a 1 N solution of sodium hydroxide. Before use the mixture is filtered through a pleated paper filter.

The flagella are stained in the following order. Onto the fixed preparation there is applied excess mordant in such a way that this should not flow off the cover glass. After 10-15 minutes the mordant is washed off with a strong stream of distilled water until all traces of the mordant disappear. The border which is formed along the edge of the cover slip from the drying mordant is carefully removed with moistened cotton wool (on a matchstick) and the preparation is again thoroughly rinsed in distilled water. During the process of mordanting the flagella increase in diameter.

The preparation is stained for 2-5 minutes in Ziehl's carbol-fuchsin diluted two-fold in water (and filtered!), rinsed in distilled water, dried in air and mounted in Canada balsam on a microscope slide. It is best to do the staining in a watch glass, placing the cover slip into the stain with the preparation side down to avoid the precipitation of a deposit.

On the basis of the arrangement of the flagella the bacteria are divided into monotrichous, amphitrichous, lophotrichous and peritrichous forms. The monotrichous bacteria are those with one polarly situated flagellum. In some bacteria the flagellum emerges somewhat below the pole of the protoplast. The amphitrichous bacteria have flagella at both

poles. The lophotrichous bacteria have a bundle of polar flagella. The peritrichous bacteria have numerous flagella over the entire body. Flagella are present in the motile cocci and sarcinas and also in non-motile bacilli.

The flagella have a sinuous form; in the lophotrichous bacteria the flagella are often plaited together into a "braid", and sometimes these bacteria may easily be mistaken for monotrichous forms.

The structure and arrangement of the flagellar apparatus is of significance in the classification of bacteria.

Fixation and staining of bacteria. In bacteriological practice the fixation of bacteria by heat is widely utilized.

A drop of sterile tap water is deposited onto a well degreased microscope slide by means of a loop; then, using a loop, some of the bacterial culture is taken from an agar slope and, touching this lightly to the drop, a thin smear is made (to the appearance of a weak cloudiness in the drop). The drop is allowed to dry out in air and, using forceps to hold the microscope slide with the dried smear, this is passed three times through the flame of the burner with the back face of the slide towards the flame. However, such coarse fixation permits determination of only the form of the bacteria and using these for Gram staining. The protoplast of the bacterial cell shrinks markedly from the heat.

Gram stain. The principle of this staining consists of the fact that some bacteria very strongly retain the iodine compounds of pararosanilines (gentian-, methyl- and

crystal-violet, and Victoria blue) and are therefore not decolorized during the treatment with alcohol; such bacteria are said to be gram-positive. Other bacteria do not retain the iodine compound and become colourless when the smear is immersed in alcohol, these are therefore said to be gram-negative. In young cultures the young cells of some bacteria 29
retain the gram-positive stain, but as they age they lose this property and become gram-negative. Because of this it is easy to make a mistake in a bacteriological control of the purity of a culture, by mistaking large young cells as an impurity, i.e. as exogenous bacteria that have accidentally contaminated the culture.

Bacteria that have been treated with acids and alkalis, as well as old bacterial cells affected by neutral formalin, lose the capacity of being stained by the Gram method. For staining bacteria in tissues and pus, aniline oil is added to the stain solutions as a mordant.

Reagents for the Gram stain

1. Gentian violet (better crystal violet) . . 1 gm
   96% ethyl alcohol . . . . . 100 ml
   glycerine . . . . . 5 ml

According to Sinev's method pieces of filter paper, cut into strips, are impregnated with the stain solution (1) and are then dried. The dried strips of stained paper can be stored for an unlimited length of time.

2. Lugol's solution:

potassium iodide	2 gm
96% ethyl alcohol	10 ml
crystalline iodine	1 gm
distilled water	100 ml

The potassium iodide is dissolved in the alcohol and then the crystalline iodine is added. After the latter is dissolved the distilled water is added in small portions. Lugol's solution should be the colour of strong tea and with no cloudiness.

3. Ziehl-Neelsen carbol-fuchsin:

basic fuchsin	1 gm
carbolic acid (crystalline)	5 gm
glycerine	a few drops
96% ethyl alcohol	10 ml
distilled water	100 ml

The basic fuchsin is thoroughly ground up in an agate mortar, adding the carbolic acid, which has been melted in a water bath, and the glycerine. Then the alcohol is gradually poured in, stirring the obtained paste, after which this is gradually diluted with distilled water. The carbol-fuchsin is stable, it should be without any cloudiness. For staining by the Gram method the fuchsin is diluted 10-fold (according to Pfeiffer).

The Gram staining is carried out in the following order. Onto the fixed smear is placed a strip of the filter paper which had been impregnated with the solution of gentian-violet (see above) and this is moistened with a small amount

of distilled water. After 2 minutes the strips of filter paper are removed and, without washing off the stain, the preparation is flooded with Lugol's solution for another 2 minutes. After this, the stain and iodine solution are poured off the preparation and, without rinsing in water, the microscope slide with the preparation is immersed vertically several times in 96% alcohol until the stain stops coming off the smear, when it is rapidly washed in distilled water. Pfeiffer's fuchsin is poured onto the smear and this stain is left on for 30-60 seconds. The stained smear is washed in water and dried in air.

Peshkov stain for spores. Onto a heat-fixed smear is poured some Löffler's blue and this is brought to the boil over the flame of a burner (5-20 seconds), it is then washed in water and counterstained with a 0.5% aqueous solution of neutral red for 30 seconds. The smear is rinsed in distilled water and dried in air. The spores are stained to a pale blue or dark blue colour, young spores - to a blackish-blue colour. The protoplasm of the cell is stained a pink colour.

Preparation of Löffler's blue: 30 ml of a saturated alcoholic solution of methylene blue, 1 ml of a 1% aqueous solution of potassium hydroxide and 100 ml of distilled water. To obtain the saturated solution 10 gm of methylene blue is taken per 100 ml of 96% alcohol and placed at a temperature of 37° for 2 - 3 days.

Aueski* method for staining spores. Onto the fixed smear is poured a mixture of equal volumes of 1% hydrochloric acid and 5% sulphuric acid, this is warmed over the flame of a burner for two minutes, without allowing it to boil, and then rinsed in water. Through a filter paper, which is placed on the acid-treated preparation, is added undiluted Ziehl's fuchsin and the preparation is again heated for 2 minutes. Then very briefly (less than a second) the preparation is immersed in a 5% solution of sulphuric acid and the decoloration is quickly halted by a rinse in water. Supplementary staining is carried out with diluted Löffler's blue (1 : 4) for several seconds and the preparation is again rinsed with water. The spores are stained a red colour, while 31
the protoplasm of the bacterial cell is a dark blue.

Romanovsky-Giemsa stain. For this purpose heat fixation over the flame of a burner is not suitable. M. A. Peshkov recommends that air dried smears be fixed with Carnoy's fluid (60 ml 96% alcohol, 10 ml glacial acetic acid and 30 ml chloroform) for 15 minutes and more. The bacterial smears may also be fixed in methyl alcohol (5 minutes), in ethyl alcohol (20 minutes), in Nikiforov's fluid (a mixture of equal volumes of ethyl alcohol and ether) for 20 minutes, or in alcohol-formalin (5 ml of commercial undiluted formalin and 95 ml 96% ethyl alcohol) for 5-10 minutes.

After fixation in Carnoy's fluid the smears are rinsed in 70% alcohol to remove the acetic acid and thoroughly dried.

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* Translator's note. Direct transliteration.

The bacteria may be stained by the Romanovsky-Giemsa method with a ready-made commercial stain (with glycerine) or with aqueous solutions of eosin (1 : 1000) and azure II (1 : 1000) according to Romanovsky - Laveran (the mixture is prepared "ex tempore"). The prepared Romanovsky - Giemsa stain and the azure-eosin mixture of Romanovsky - Laveran are slightly diluted with alkaline distilled water (pH 7.2). For alkalizing the water M. A. Peshkov recommends a 1% aqueous solution of sodium carbonate (Na_2CO_3), previously staining this with a 0.5% solution of neutral red to a weak pink tinge. The sodium carbonate is added drop by drop till the stained water becomes colourless; this water should not become pink again after the lapse of three minutes. In practice this method of alkalizing the water for diluting the stains, used for staining the smears of the bacterial cultures and the blood smears, is very convenient.

1 drop of the commercial Romanovsky - Giemsa stain is taken per 1 ml of the alkalized water, while the azure-eosin mixture of Romanovsky - Laveran is prepared from the basic aqueous solutions of azure II (1 : 1000) and yellowish eosin (1 : 1000) in the following proportions:

azure II . . . . . 4 ml
yellowish eosin . . . . . 6 ml
distilled water (pH 7.2) . . . . . 6 ml.

The staining of the smear is carried out in the following manner. The microscope slide is placed with the smear downwards into a Petri dish onto two glass splinters (or into a large watch glass) and the diluted stain is poured in under 3

the slide in such a way that it covers the whole of the smear. This procedure protects the smear from precipitation of the stain. Staining lasts from 1 to 24 hours. For differentiation of the structure of the bacterial cells the smears are washed in 70% alcohol, with microscopic control being applied. Better differentiation of the basophilia of the cytoplasm is achieved by treating the stained smear with a 5% aqueous solution of tannin.

The polychromic staining according to the method of Romanovsky - Giemsa - Laveran shows up the structure of bacterial cells in a still more demonstrative manner with the supplemental staining, according to the method of M. A. Peshkov, with an acidified solution of light green (100 ml of a 0.25% aqueous solution of light green and 1-2 drops acetic acid) with a subsequent treatment with tannin. For this purpose a part of the smear is covered with a drop of the light green solution for a few seconds (the duration of the exposure depends on the age and species of the culture) and, without washing, the drop is removed with filter paper; then onto the smear is poured a 5% solution of tannin. The differentiation is controlled under the microscope. After differentiation is completed the tannin is removed with filter paper. As a result of the partial counterstaining of the smear the differentation of the cells occurs in a particularly demonstrative manner at the border of the supplementarily stained area.

The method proposed by M. A. Peshkov not only intensifies the contrasting of the cell structure but also permits one to differentiate living bacteria from dead and dying cells.

Young bacterial cells from a 4-10-hour culture have a clearly basophilic cytoplasm and darkened nuclear elements, but in an 18-hour culture the cytoplasm of the cells is oxyphilic. Therefore, after the counterstaining with the light green solution the nuclear elements of the cells, which are stained a red-violet colour, are very clearly distinguishable against the green background of the cytoplasm. In the dying cells there is evident a reduction in the amount of nuclear substance, while in the dead cells this substance disappears and the cells become empty, i.e. with only cytoplasm which is stained a green colour.

The nuclear structure of the young basophilic bacterial cells, stained with Romanovsky - Giemsa, is revealed under microscopic examination with a blue-green filter, since the latter is the complement of red.

Staining of capsules. The capsules of some 3
bacteria are best revealed by a saturated aqueous solution of Congo red (Peshkov, 1955), if the background is contrasted with a blue-green filter during microscopic examination. The saturated solution of the stain is prepared by boiling 0.5 gm of Congo red in 10 ml distilled water. One drop of the stain is mixed on a microscope slide with the bacterial suspension, which was taken up by means of a loop; the mixture obtained is transferred with a loop onto another microscope slide and is pressed down strongly with a cover glass (by the convex side!), drawing up the excess liquid with filter paper. The microscopical picture shows the capsules appearing in the form of an aureole around the bacterial cell. The application of

the saturated solution of Congo red provides an opportunity for distinguishing living from dead bacteria, since in the latter the cytoplasm is oxyphilic.

Identification of isolated strains of bacterial cultures

For the identification of the species affinity of the strains of the pure bacterial cultures M. A. Peshkov proposed a scheme of bacteriological investigation which permits a strict systematization of the material obtained on the basis of the morphological and biochemical properties of the microbes. For each isolated strain of bacterial culture there is set up a record card (see Appendix). This serves as a passport for the isolated microbe, in which passport are given its origin and characteristic morphological and biochemical features, on the basis of which the microbial species is identified.

Both the morphological as well as the biochemical properties of bacteria are very variable, which depends on the conditions of the medium in which they are cultured. Therefore it is fairly difficult to identify a bacterial species. Many species of bacteria are polymorphic during the process of development. This is noted especially during the first hours of their development, when there appear in the culture giant basophilic young cells. Therefore, in investigating the morphology it is important to know precisely the age of the culture. Thus, a 2-6-hour bacterial culture differs markedly in its morphology from a 24-hour culture, while the latter differs from 48-hour and older cultures.

The biochemical properties of a bacterial culture also vary depending on its age. In 3-4-day and older cultures some enzymatic properties may be lost. Therefore, enzymatic properties are studied only in 18-24-hour cultures.

Morphologically similar species of bacteria differ physiologically in their complement of enzymes. The enzymatic properties of bacteria are revealed by culturing them on special nutritive media, containing various sugars, alcohols, proteins, fats and other substances. A series of such various special differential-diagnostic nutritive media is called a "variegated series". As a result of inoculations of the investigated cultures on the media of the "variegated series", the bacterial strains are identified on the basis of their biochemical properties in accordance with the morphological data.

Temperature optimum for bacterial growth. The optimal temperature is first determined on the basis of the intensity of the growth of the culture on MPB. For this the culture is inoculated on liquid MPB medium and placed in a thermostat at different temperatures: 10, 15, 20, 25, 30 and 37°. Subsequent observations on the biological properties of the bacterial cultures are conducted at that temperature at which the most luxuriant growth on MPB is obtained.

Morphology. In the morphological investigations smears of 24-hour cultures are stained with the Gram stain, and determinations are made simultaneously of the form of the cell, its size and relationship to the staining. In order to

expedite the staining of smears of a large number of cultures, onto one microscope slide there are placed 3 thin smears in the sequence in which the strains of cultures are placed in the test tube racks. Inscriptions on the microscope slides are made with a wax pencil at one edge on the clean side of the slide, so that during the differentiation of the smear the inscription is not dissolved in the alcohol. Therefore it is more convenient to make use of microscope slides with a frosted surface at one end for making inscriptions with an ordinary pencil. After microscopic examination, the immersion oil is removed from the preparation by immersing the microscope slide in xylol. This permits one to repeatedly examine a wanted preparation after destaining and staining with other stains.

Particular attention is paid to the study of the flagellar apparatus as an important morphological feature in the systematics of bacteria.

The capacity for spore-formation should also be attentively verified. In some species of bacteria spores are easily found in old agar cultures, while in others they may be found after prolonged culturing on the "semi-starvation" solid media of Gorodkova, Tarr and others. Smears are prepared from cultures of various age depending on the species of microbe. The quality of the staining of the spores depends on the time of exposure to the mordant.

The capacity for capsule-formation in bacteria is revealed by growing the cultures on a normal food medium (MPA).

Capsule-formation is characteristic not only of the pathogenic bacteria but also of many saprophytes. Capsule-forming bacteria produce a slimy growth of the culture on MPA, but the formation of slime is also possible in non-capsulated bacteria.

Character of the growth of the culture on MPA. Many species of bacteria are characterized by the specific characteristics of the growth of the stroke culture on an MPA slope or of the growth of the colonies.

On an agar slope (the slopes are made on the day of inoculation!) the bacterial culture is inoculated by a stroke from the bottom upwards, immersing the loop in the condensed water that has accumulated at the bottom of the tube. On the following day and subsequently over the course of a week observations are made of the growth or absence of growth of the culture on MPA. The following characteristics of the growth on the agar slope are distinguished: the intensity of the growth - meagre, average, good, plentiful with distinct colonies or confluent; the character of the growth - filiform, divaricate and rhizoid; the surface of the growth - dry or moist; pigment formation - colour of pigment, soluble or insoluble in the medium.

In Petri dishes on MPA 1-3-day old colonies are distinguishable by the following features: by dimensions - small, large; by the character of the growth - round, divaricate, rhizoid; by the density - transparent, cloudy, dark; by the pigment - coloured, colourless; by the surface - convex, flat, concave, smooth, rough, wavy, patterned, grainy, with papillae,

pitted, dry, moist: by the margin - even, wavy, lobate, pitted, villose, in curves, creeping.

On the data card for the isolated strain of bacterial culture a schematic illustration is made of the structure of the colony in surface view (under the microscope: X8 ob., X5 oc., without condenser) and in profile. A precise representation of the character of the structure of a colony is given by a photograph, made at the above-mentioned magnification (artificial light source with an open diaphragm of the illum[illegible]ator). Sometimes, especially with unfavourable conditions of culturing, disintegrations (dissociation) are possible: there are formed smooth colonies, S-forms, ragged forms and R-forms, which differ not only in their morphological but also in their enzymatic properties. 36

Growth of the culture on MPB. The results of the inoculation of a 1-day old bacterial culture into MPB are recorded on the following day and after 3-4 days. A note is made of the absence of turbidity of the medium or of an even or layered turbidity. Attention is paid to the character of the sediment - smoky, granular or flocculent. Dissolved pigment may be found to occur and there may be formed on the surface of the bouillon a film or a ring on the glass of the test tube.

Growth of the culture on MPG. A one-day old culture on an agar slope is transferred by a stab of a platinum needle in the center of a column of MPG to the bottom of the test tube. with inoculation with a bacteriological loop the pattern of

the growth is distorted, since a bubble is formed within the medium. The results of the inoculation are recorded 3 times: on the 1st, 10th and 20th days, noted by the Roman numerals I, X, XX. Liquefaction is noted by the plus sign, the absence of liquefaction - by the minus sign. The character of the liquefaction is schematically sketched: with the form of a nail, cup, funnel, stocking or in layers. Growth of the culture without liquefaction is also noted: on the surface, deep down along the puncture, arborescence from the puncture.

Fermentation of milk. A one-day old agar bacterial culture is taken up with a loop and inoculated into Minkevich medium (milk with litmus) in test tubes. The results are recorded on: the following day, and days 10 and 30. Shifts in the pH value are determined by the change in colour of the medium. Minkevich medium is opaque and has a violet colour (neutral reaction). As a consequence of the metabolism of the bacteria the inoculated medium acquires a shade ranging from a reddish-violet to red colour in step with the formation of acid or from a bluish-violet to blue in step with the formation of alkali. Some species of bacteria do not change the medium. In addition to the change in colour, as a result of the vital activity of the bacteria there may occur a bleaching (reduction) of the medium, peptonization (the medium becomes transparent as a result of the dissolution of the casein), coagulation (curdling) under the influence of acidification or of the rennet enzyme. The acid coagulate of casein is soluble in alkalis and can be peptonized, while the rennet coagulum, in contrast, is insoluble in alkalis and contracts on standing; above this is formed a transparent liquid.

All of the observed changes are recorded by conventional designations: K (acid), Shch (alkali), P (peptonization), KS (acid coagulate), SS (rennet coagulate), N (neutral), NK (weak acid), NShch (weak alkali), R (reduction). There are often observed layered changes, for example: there is formed an acid coagulate, the lower layer of which is decolorized. In such a case a fractional notation is made ($KS\frac{K}{R}$); in another case there may occur a peptonization, and here there will be a weak alkali in the upper layer, while in the lower layer there is a neutral reaction; then the notation will have the form ($P\frac{NShch}{N}$).

Growth on potato. A one-day old agar bacterial cultureis inoculated by means of a loop, making streaks on the slanted surface of a piece of potato in a test tube. The results of this inoculation are recorded twice: on the 10th and 30th day, at which time is noted the intensity and character of the growth, and also the colour of the culture, which differs from the colour of the agar culture. Attention is paid to the dampness, dryness, convexity of the growth of the culture and to the breakdown of the piece of potato itself.

Fermentation of carbohydrates on Giss medium. A one-day old agar bacterial culture is inoculated with a loop onto solid Giss medium (this is more convenient than into the liquid medium with floats). The results of the vital activity of the culture are recorded on the following day and on the 6th day. Acid- and gas-formation are noted.

The Giss medium with Andrade indicator is colourless. With the formation of acid the medium becomes red; in the

case of gas-formation the bubbles of gas rupture the agar and produce a froth on it. M. A. Peshkov proposed a convenient method for recording all of the possible cases of change in the medium by means of symbolic designations:

no change in medium	−
acid in all layers of the medium	+
acid in upper layer and no change in lower	±
weak acid	†
weak acid in upper layer and no change in lower	ŧ
strong formation of gas with acid	⊕
medium formation of gass with acid	ȸ
little formation of gass with acid	⁺

38

Relationship of culture to oxygen. The inoculation of a one-day old agar bacterial culture with a loop onto Klodnitskii medium or onto Peshkov's modification of the Klodnitskii medium with neutral red (or bromothymol blue) provides an opportunity for determining the oxygen requirement of a given species of bacteria. Aerobes develop only in the upper layer of the medium, facultative anaerobes - throughout the whole thickness of the medium down to the bottom of the test tube, anaerobes - only in the lower layer of the medium, clearly limiting the zone of growth. The addition of coloured indicators (neutral red or bromothymol blue) permits one simultaneously to establish the formation of acid and gas in the case, for example, of the fermentation of glucose by bacteria of the intestinal group, Aeromonas punctata and others.

The medium with neutral red has the colour of port wine, in the presence of oxygen it acquires a raspberry colour. In the case of reduction neutral red gives a yellow-green fluorescence.

Formation of hydrogen sulphide and ammonia. M. A. Peshkov proposed a simple method for indicating hydrogen sulphide and ammonia: the application of paper impregnated with lead acetate and of litmus paper. For this purpose MPB is inoculated with a one-day old agar bacterial culture and under the cotton-wool stopper of the test tube are placed the indicator papers: for indicating hydrogen sulphide - a strip of filter paper impregnated with 0.5 - 1% lead acetate, while for indicating ammonia - red litmus paper. It is recommended that a rubber cap (fingerstall) be placed over the stopper.

In the case of the production of hydrogen sulphide the lead paper darkens to a metallic lustre, while with the formation of ammonia the litmus paper turns blue.

Reduction of nitrates and nitrites. The denitrifying capacity of bacteria is determined by the method of M. A. Peshkov, by culturing these on MPB, containing 0.5% potassium nitrate. A preliminary check is made to test the absence of nitrates in the MPB and the purity of the potassium nitrate. Then the normal inoculation is made.

For determining denitrification the following reagents are required: 39

soluble starch	1.0 gm
potassium iodide	0.5 gm
distilled water	100 ml

The starch is dissolved by boiling and, after this is cooled, the potassium iodide (unstable reagent) is added.

To acidify the reaction there is added to a 3-4-day old culture (5 ml) 0.5 ml of chemically pure 10% sulphuric acidand 0.5 ml of the starch solution with potassium iodide. The sulphuric acid may be replaced by 20% acetic acid. A positive reaction, the turning blue of the mixture in the test tube, is recorded by a plus sign; this reaction indicates the transition of the nitrate salt to the nitrite. A negative reaction, no turning blue of the mixture in the test tube, is recorded either by a minus sign or by two pluses, since in the former case (-) the bacteria of the given species do not assimilate nitrate salts during the process of their vital activity, while in the latter case (++), in contrast, the bacteria decompose not only the nitrates but also the nitrite salts. In order to establish this decomposition (the latter case), there are added to the test tube, in which the mixture did not turn blue, chemically pure filings or granules of zinc. In an acid medium zinc reduces nitrates to nitrites. Therefore, if the colourless mixture turns blue, this will indicate that the culture was devoid of a denitrifying capacity, while if the mixture does not turn blue, this will indicate further decomposition of the nitrites, i.e. a greater denitrifying capacity of the culture (++).

Formation of indole. In a 4-day old bouillon (Khottinger bouillion) culture the formation of indole is determined by the Salkowski method or by the Ehrlich method. When utilizing normal MPB the quality of the peptone is checked for indole.

According to the Salkowski method, to the culture in the test tube is added 0.5—1 ml amyl alcohol, this is shaken and there is poured in 1 ml of an aqueous solution (1 : 1000) of potassium or sodium nitrite, the medium is acidified with 1 ml of concentrated, chemically pure sulphuric acid. A pink colour of the alcohol layer indicates a positive reaction.

According to the Ehrlich method, to a 4-day old culture there are carefully added, along the wall of the test tube, a few drops of an alcoholic solution of paradimethylamidobenzoaldehyde with concentrated hydrochloric acid. If 40
the reaction is positive, then during the course of one minute the upper layer of the medium takes on a cherry-red colour.

Composition of Ehrlich's reagent:

paradimethylamidobenzoaldehyde	1.0 gm
96% ethyl alcohol	95.0 ml
concentrated hydrochloric acid	20.0 ml

Voges-Proskauer reaction (acetyl-methyl-carbinol) and reaction to methyl red. These reactions are of considerable significance in the identification of isolated strains of bacterial cultures. In the course of these reations there are clearly manifested differences between bacteria of the coli-typhi type and saprophytes. The formation of acetyl-methyl-carbinol and the reaction to methyl red are determined

on a 4-day old culture on Clarke medium. The culturing on this medium is carried out at a temperature of 37°. On the 4th day of culturing a part of the culture is poured off into an empty test tube for the reaction with methyl red, while the remaining portion is retained for the acetyl-methyl-carbinol reaction. The latter is determined by a 10% solution of potassium hydroxide, which is added to the culture in equal volume. The reaction with the alkali proceeds well at room temperature (18-20°), it is read after 2-6 hours on the same day (preferably in day-light). With a positive reaction there is formed an eosin-red coloration of the medium (in layers). To the remaining portion of the culture which was poured off into a test tube are added several drops of an alcoholic solution of methyl red. A red coloration of the medium is recorded as the negative reaction (-), while a yellow coloration is recorded as the positive reaction (+).

The solution of methyl red is prepared by dissolving 0.01 gm of methyl red in 30 ml 96% ethyl alcohol and adding this to 50 ml distilled water. This may be stored for a long time.

Decomposition of fat. Jelled MPA with fat in Petri dishes is divided into several sectors, cutting out any fissures in the agar with a sterile scalpel. On the sectors obtained stroke inoculations are made of one-day old agar bacterial cultures. The number of the strain is inscribed on the dish. After 18-24 hours of culturing the bacterial colonies that have grown up are flooded with a saturated solution of copper sulphate; after 5-6 minutes this is carefully poured off the

agar. In this case the bacterial colonies that have decomposed fat are stained a light blue colour as a consequence of the reaction between the free fatty acids and the $CuSO_4$. 41

Hydrolysis of starch. Gelled MPA with starch in Petri dishes is divided into several sectors, in the same way as the agar with fat, and also inoculated with culture in a stroke fashion in accordance with the number of the strain. After 18-24 hours of incubation in a thermostat the culture which has grown on the surface of the agar is flooded with Lugol's solution. In the case of hydrolysis of the starch, around the strokes there is formed a brown or light-coloured zone on the black-violet background. The intensity of the hydrolysis is determined by the width of this zone and by its colour.

Identification often requires supplementary information on features of the isolated strains for a complete characterization of these according to the attributes which are mentioned in keys to bacteria (for example, in Bergey). The investigation of the biochemical properties of isolated strains of bacterial cultures is facilitated by a strict sequence of the inoculations in the media of the "variegated series", if the latter makes up a complete set in accordance with the points mentioned on the record card (passport) of the strain. If there is obtained a luxuriant growth of a day old bacterial culture on an agar slant, then this amount of bacterial mass is generally sufficient for inoculation into several test tubes with media of the "variegated series" (only adequate technical skill is needed).

Determination of the pathogenic properties of bacteria on fishes

According to Koch, the true causative agent of a given infectious disease is that microbe, the pure culture of which causes a similar infection experimentally, i.e. the pathogenic property of the isolated microbe should be specific. Therefore, from the bacterial strains that have been isolated from a diseased organism, especially from the blood of the heart and other internal organs, there should be revealed precisely that microbe which would possess the specific properties of causing the disease. This is achieved by the experimental infection of fishes, for which it is desirable to have fish of the same species and of the same age.

The experimentally infected fish are held in aquaria or in special tanks, in which the water temperature and flow are regulated in accordance with the natural conditions. Thus, for example, the temperature optimum for the pathogen of furunculosis of salmonids is 10-15°, while trout require strongly flowing cold water. The development of the infectious process in the organism of the fish is also possible at a higher temperature and therefore experiments are set up at various temperatures of the water. Thus, in setting up the experiments one should be guided not only by the optimal temperature for the development of the microbe on the artificial nutrient medium in the thermostat, but also by the water temperature at which the given disease of the fish appeared in the body of water. However the main role in the experiment is played by the hydrochemical regime that is characteristic of the given species of fish.

The fish are experimentally infected with the test cultures or pathological material in varying doses. The infection is accomplished by way of injection subcutaneously, intramuscularly, intraperitoneally, into the brain, or by a catheter through the mouth and anus; infection is carried out by scarification and contact, by placing healthy fish together with diseased fish or by placing traumatized and non-traumatized fish into an aquarium, into which is introduced the test bacterial culture or pathological material from dead diseased fish. The results of the infection are considered by the statistical method of Rid and Nench*, by this means the lethal dose (LD_{50}) is revealed.

In the determination of the lethal dose the clinical and pathologico-anatomical picture is taken into consideration; the observed changes are defined in greater detail by histological studies, which together with the hematological indices provide a picture of the specific pattern of the course of the infectious process, notwithstanding any superficial stereotypy.

The reactivity of the fish organism to the effect of the the bacterial infection is studied directly on fish of the same species. It is true that it is not always possible to utilize fish of the same species, in which the disease was found under natural conditions, for experimental infection in aquarium conditions. Therefore, as models for the cold-loving fishes we recommend the trout, while for the warm-loving fishes the recommended models are the crucian carp, carp, tench, orfe and ide.

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*Translator's note. Direct transliteration. Possibly Reed, Read or similar phonetic equivalent.

The clinical symptoms and pathologico-anatomical changes in the fish are the result of the reflex action of the organism against the action of the specific infectious irritant. In establishing the pathogenic properties of bacteria to fish contradictory results may be obtained. This depends on the individual reactivity of the fish organism, the virulence of the microbe, the amount of culture introduced, the site and method of inoculation, and the general conditions of the experimental set-up (temperature, nutrition, aeration etc.).

In one case the organism of the fish of a given species may be unresponsive to the test infection because of an inherited (natural) immunity, in another this may be so because of an acquired immunity. Consequently, the correct selection of the experimental fish is of considerable significance.

A bacterial culture in laboratory conditions of culturing on artificial nutrient media may partially or completely lose its virulence. However by a repeated passaging of the culture through a living organism (fish) it is sometimes possible to re-establish the virulence. In re-establishing the virulence of a strain of bacterial culture, its purity is controlled and checks are made of its biochemical properties (on media of an abbreviated "variegated series"), since otherwise the initial culture may easily be lost during the passages.

The quantity of the bacterial culture which is required for inoculation is determined by a preliminary test; it depends on the virulence of the strain and on the weight of the test fish. The culture is always injected in one and the

same place, shown in Figure 3. If the infectious agent is unknown, then usually there are simultaneously employed various methods of inoculation, since it is difficult to foresee in advance where the development of the process will give a more specific picture of pathological changes. The application of the infectious agent to fish through a mechanical trauma may evoke a local inflammatory process in the dermo-muscular region, while if the pathogen gets into the blood the process may be evoked in a distant organ or there may be a bacteremia, depending on the specificity of the microbe.

In the examination of the fish, attention is paid to the following superficial features.

The behaviour of the fish: does it hold itself near the bottom or at the surface of the water, swallowing air in the presence of an adequate oxygen content of the water; is it lying on its side on the bottom or at the surface of the water almost without movement; is it swimming in a vertical position with the head down or with the head up; has it lost coordination of movement, swimming in a circle or in a spinning manner; does it react to contact with a human hand; is the gill breathing slowed or accelerated? 44

Changes in pigmentation: a general darkening or lightening of the skin of the body; a localized position or local depigmentation in the form of patches on the skin of the body; a general, local or scattered pigmentation that is not characteristic of the fish in nature (yellowing, reddening, greyish tinge).

Рис. 3. Места инъекций рыб:
а — внутримышечно или подкожно; б — внутрибрюшинно.

Figure 3. Sites of injection on fish:
a - intramuscular or subcutaneous; b - intraperitoneal.

Hyperemia and hemorrhages: dilatation of the blood vessels and capillaries of the skin and eyeball; hemorrhages (punctate and diffuse) in the skin and eyeball.

Edema: general or local edema, exophthalmos, bristling of the scales, ascites; local hemorrhagic swelling of the dermal tissue.

Infiltrations, abscesses: local inflammatory swellings on the body with the formation of abscesses; infiltrations in the region of the anus and inflammation of the anus.

Ulcer formation: cutaneous erosions with a flat bottom; necroses; granulations and cicatrizations.

During pathologico-anatomical dissections attention is paid to: the abdominal cavity - dry, moist, with a colourless, bloody, purulent, cloudy or jelly-like exudate; liver - colour, hyperemia or anemia, congestive consolidation, friability, mosaic patterning, increase or decrease in volume; spleen - colour, mosaic patterning, increase or decrease in volume; gall bladder - increase or decrease in volume, transparency or cloudiness and colour of bile; gonads (of males and females) - hyperemia, stages of maturity, degeneration; gastro-intestinal tract - contents (food mass, pus, bloody mucus, colourless mucus), hyperemia or anemia of the walls, swelling of the mucosa, ulcerations, erosion, perforation; kidneys - hyperemia or anemia, congestive consolidation, mosaic patterning, friability; heart - transparent or cloudy pericardial fluid, hemorrhages in muscular wall; brain - hyperemia of vessels and hemorrhages et al.

In studying the blood smears and impressions of the internal organs the presence of bacteria is noted. Then there is established the cytological picture of the blood elements and of the changed internal organs.

The organism of the fish reacts to the irritation caused by an infectious agent by the formation of some or other cellular elements of the blood and by a change in the protein composition of the blood serum. Therefore, in ichthyopathology determinations are made of the deviations which occur in the leucocytic formula, and the blood serum proteins

are fractionated by paper or gel electrophoresis. The quantity of the protein fractions in the blood serum of fishes depends on the physiological state of the organism, especially in pathological cases. Changes occur in the quantitative relationship of the proteins (albumin and globulins).

Serological studies for the identification of bacteria and the diagnosis of infectious diseases 4(

Some species of bacteria, notwithstanding a similarity of their morphological and biochemical characteristics, are distinguishable by their complex of antigens - special substances which produce the corresponding defensive antibodies in the organism of the animal in the course of natural diseases or artificial infection. In connection with this, in the serodiagnosis of infectious diseases and in the identification of the antigenic relationships of different bacterial strains there are widely employed the immunological reactions of agglutination and precipitation. However in the diagnosis of fish diseases it should be taken into consideration that the serum of fish may give a group reaction of agglutination. To avoid erroneous conclusions, the agglutination reaction is carried out after adsorption, according to Castellani, which removes extraneous antibodies from the serum, and the cross reaction is set up.

The ring precipitation reaction has a high sensitivity; this reaction permits the detection of insignificantly small quantities of antigen (in dilutions of 1 : 1,000,000).

The agglutination reaction in the diagnosis of infectious diseases of fishes is in essence in no way different from the agglutination reaction proposed by Widal in the diagnosis of typhoid. Serving as antigens in the reaction are the strains of bacterial cultures isolated from the organism of the diseased fishes; the antibodies used are the antibodies from the serum of the diseased fishes, from which these strains of bacterial cultures were isolated, and antibodies from the sera of diseased or superficially healthy fish of the same population. At the present time there are no standard sera and ready-made diagnosticums of pathogens of fish diseases. The assumption that a fish from a natural body of water has already had the given disease or is healthy cannot be accepted as being certain. Therefore the results of the agglutination reaction may be only relative. In order to make sure of the reliability of such data, the fish are infected and immunized in aquarium conditions, to obtain the immune sera from these. For studying the antigenic relationships of bacterial strains there are employed rabbit sera which have been immunized by the studied strains of bacterial cultures.

For obtaining immune sera from fishes immunization is carried out intraperitoneally with an inactivated bacterial suspension of the test strain at a dose of 0.2 ml per 50 gm weight of fish in three injections, with an interval of 10 days between the first and second injections and the third injection following after one month. The bacterial suspension is prepared according to the standard of 1 milliard (10^9) cells

in 1 ml from a washing of a day-old agar culture. Only smooth S-forms are selected. The dose for the first injection is halved - 0.5 milliard, the second dose is 1 milliard and the third is 0.5 milliard. This scheme of immunization is recommended for the carp at a water temperature of 18-20°. Aeromonas punctata and Pseudomonas fluorescens, representatives of the bacterial flora of fishes, have 2 antigens: a somatic thermostable antigen and a flagellar thermolabile antigen. The specific somatic O-antigen gives a fine-grained agglutinate, the flagellar group H-antigen gives a coarse flocculent agglutinate. The agglutination reaction is set up with the specific somatic O-antigen, which is prepared by heating the bacterial suspension obtained from the washing of the day-old agar culture. The bacterial suspension is prepared in a 0.65% physiological saline for fishes and heated at 56° for 30 - 45 minutes in an ultrathermostat. The suspension should be without any clusters, but if these are formed during inactivation, it is vigorously shaken and filtered through a single layer of filter paper or centrifuged at 100 r.p.m. for 5 minutes.

A particular temperature optimum is necessary for the process of the formation of antibodies in fishes of different species. Thus, for example, in the carp and the bream a high antibody titer, greater than 1 : 10,240, is produced with a threefold immunization and at a water temperature of 18-20°, while in the burbot this occurs within the limits of 7-10°, in accordance with the temperature optimum for the metabolism of these fishes.

Rabbits are immunized by injecting these intravenously (into the auricular marginal vein), in doses of 0.5 ml, with inactivated bacterial suspension twice weekly for six weeks, gradually increasing the dose from 1 milliard to 2 milliard cells per 1 ml. Before injection the rabbit is immobilized in a sling or secured in a special cradle. Holding the ear of the rabbit in the left hand the site of the puncture is lightly rubbed with xylol and rinsed with alcohol. A helper applies pressure with his finger to the marginal vein at the base of the ear. The needle of the syringe is introduced into the vein in the direction of the movement of the blood (towards the heart). At the moment of the penetration of the needle into the lumen of the vein the helper removes his finger and the fluid freely enters the vein. If, however, the fluid enters with difficulty, this means that the needle has entered the perivascular tissue. As a result of an unsuccessful injection the vein becomes unsuitable for further utilization. Generally, injections are made into the vein of one ear, while blood is taken from the vein of the other ear. After the injection is completed the needle is removed and sterile cotton-wool is applied to the site of puncture.

To obtain serum, blood is taken from the heart or from the caudal vein. In the latter case the capillary end of a Pasteur pipette is introduced with a rotating movement into the caudal peduncle from the ventral side at the base of the last ray of the anal fin until the resistance of the backbone is encountered. With an adequately worked out technique blood taps from a single fish may be taken several times, to check the dynamics of antibody accumulation.

In rabbits, blood for obtaining antiserum is taken from the marginal vein of the ear or, in the case of complete exsanguination, from the carotid or femoral artery. Before taking the blood from the ear the skin is wiped with xylol and alcohol as a means of disinfection and to dilate the vein. Then a longitudinal cut (2-3 mm in length) of the vein is made with a sharp scalpel and the blood is collected in a sterile test tube.

On the basis of the known antiserum, by the agglutination reaction are established the causative agent of the disease and the antigenic relationships of the isolated bacteria, while on the basis of the known antigen the diagnosis of the disease can be made.

It is recommended that the agglutination reaction be set up with microbes isolated from diseased fish. The serum is taken from diseased or previously diseased fish from the same body of water.

Setting-up the agglutination reaction. The agglutination reaction is set up in serological test tubes (100 mm in length, 8 mm in diameter), several rows of which are placed in a rack, into each of these is poured 0.5 ml of physiological saline. The initial solution of serum is prepared at a dilution of 1 : 5, for which to 1 ml of serum is added 4 ml of physiological saline. In order to obtain a serum dilution of 1 : 10, to the first test tube in the row there is added 0.5 ml of the prepared serum, of a 1 : 5 dilution, to the equal amount of physiological saline; then 0.5 ml of the mixture in the first test tube is transferred into

the second and there is obtained a serum dilution of 1 : 20, etc. By successively doubling the serum dilution, the dilution is taken to 1 : 1280 or 1 : 2560. The last test tube in the row is the control, in case of the appearance of a spontaneous agglutination, and therefore it is left free of serum. Since the serum by itself may also produce a precipitate, one test tube with a serum dilution of 1 : 5 is kept as a control. Then to the diluted serum in the test tubes is added the antigen - the bacterial suspensions in equal numbers of drops: to 0.5 ml of the diluted serum are added two drops of suspension (2 milliard). 49

In this way, having 1 ml of an a priori known serum in a dilution of 1 : 5, there may be set up agglutination reactions with ten different bacterial suspensions for establishing their antigenic relationship, provided that there are no extraneous agglutinins in the serum. However, in the presence of extraneous agglutinins there is applied Castellani's method, based on the adsorption of these agglutinins by various microbes, the antigens of which are equivalent. For revealing group agglutination of various strains of bacterial cultures the reaction is set up with one and the same serum, extracting the homologous agglutinins one after another with subsequent centrifugation and removal of the precipitate.

The agglutination reaction with a known antigen and unknown serum is set up in a similar manner.

The specificity of the agglutination reaction is determined by the cross reaction. For example, if one has two microbial antigens A and B and two homologous sera A and B,

then these are combined in the following manner: antigen A + serum A; antigen A + serum B; antigen B + serum A; antigen B + serum B.

The agglutination reactions with immune rabbit sera are set up in a thermostat at a temperature of 37°, while those with immune fish sera are set up at room temperature. The reactions are read with the aid of an agglutinoscope on the following day (after 18-24 hours). The degree of the intensity of the reaction is noted on a 4-point scale using plus signs (+, ++, +++, ++++), an indistinct rection is noted by a plus-minus sign (±) and a negative reaction by a minus sign.

Precipitation reaction. The ring-precipitation reaction is one of the most sensitive serological reactions. As the antigen is used the complete antigen (according to Buaven and Mezrobian) or the hapten. If one has preparations of the complete antigen or hapten from a known pathogenic microbe and the blood serum of the diseased or recovered fish, then a diagnosis of the disease may be established. If, however, one has preparations of the antigens from a culture of an isolated bacterial strain, the relationship of which to a given disease is unknown, one can establish the causative agent of the disease by the formation of precipitins in the serum of the diseased or recovered fish.

Obtaining complete antigen. To obtain complete antigen a large number of bacterial cells of the culture are required. These are cultured in special flat flasks ("mattresses") on MPA. 18-20-hour cultures on "mattresses"

are washed off with sterile physiological saline and (the washings) are centrifuged at 8000-10000 r.p.m. The precipitate is washed three times by centrifugation in water and is once more suspended in distilled water in such a way that there be 200 mg of the wet microbial cells in 1 ml of the suspension. After cooling the suspension obtained to 0° there is added to it 0.5 N trichloracetic acid in the ratio of 1 : 1 (Sharova, 1936). This mixture is vigorously shaken for several minutes and placed in a cold room at a temperature of 0° for 3 hours, with periodical shaking. After the 3-hour extraction the mixture is centrifuged at 8000 - 10000 r.p.m. until the bacteria are completely precipitated. The slightly opalescent supernatant fluid (extract) is subjected to a twice repeated dialysis to remove acids and other organic and mineral substances. For the dialysis a collodion bag may be prepared from a 5% solution of nitrocellulose in glacial acetic acid or ether.

The first dialysis is conducted in a cellophane or collodion bag for 48 hours, placing the bag with the extract in running tap water. Before the dialysis, a check is made of the integrity of the bag and of its impermeability to protein by the biuret reaction.

The second dialysis is carried out in the cold room, having previously acidified the extract with hydrochloric acid to the degree of reddening of the methyl red indicator. The bag with the extract is placed in a large vessel with distilled water for 24 hours (1 liter of distilled water per 10 ml of extract).

If there is a precipitate in the dialysis-purified extract, the extract is centrifuged and the precipitate is discarded. The extract is sterilized by filtration through a Chamberland L_3 filter (or a No. 2 membrane filter). The sterile extract retains its biological properties for several weeks when kept on ice.

Thus, the prepared extract is a solution of a polysaccharide complex. This extract, which was called complete antigen by Buaven, is utilized for the ring-precipitation reaction and injection into animals.

In preparing the complete antigen in the form of a dry preparation the dialyzed extract is evaporated under a vacuum at room temperature, or the dialyzed extract is slightly acidified with hydrochloric acid and, after it is first concentrated in a vacuum, there is added to it absolute ethyl alcohol or acetone to a concentration of 68% (2-3 of the volume of the alcohol*). For a better precipitation of the antigen, ethyl alcohol saturated with potassium acetate is utilized. The precipitated deposit of antigen is centrifuged or filtered, it is then washed with alcohol and ether, and dried in a vacuum exsiccator over phosphorus anhydride. The yield of the preparation in the dry form comprises about 3.5%. The dry powder of the antigen may be stored in ampoules in a refrigerator for a fairly long time. Before use the antigen is dissolved in water.

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*Translator's note. sic. pbly. 2-3 times the volume of the alcohol.

Obtaining the hapten. The Moscow City Institute of Epidemiology and Bacteriology has worked out a fast method for the diagnosis of intestinal infections. Lying at the basis of this method is the specific property of hapten, which is a polysaccharide component of the somatic antigen of gram-negative bacteria. Hapten produces a precipitate in combination with the homologous antibodies of the blood serum.

Hapten is obtained from an extract of bacterial suspension by the following procedure (Predtechenskii, 1960): 4-5 ml of an 18-20-hour bacterial culture, inoculated in a Petri dish on MPA with a spatula so as to obtain a compact growth of the bacteria, is washed off with a 10% solution of acetic acid into a test tube. The washing is boiled in a water bath for 30 minutes to 1 hour. Then the suspension is filtered through asbestos wool. The transparent colourless filtrate that is obtained is neutralized with a 20% solution of sodium carbonate in the presence of bromothymol blue indicator. If a precipitate is produced during alkalization, the filtration is repeated. A colourless transparent filtrate is obtained and this is the hapten.

Setting-up the ring-precipitation reaction. The ring precipitation reaction is set up in narrow test tubes (3-4 mm in diameter). In contrast to the agglutination reaction, this reaction is carried out not with diluted but with entire serum. From the solution of the complete antigen or hapten there are prepared several dilutions: 1 : 1000, 1 : 2000, 1 : 4000 etc. The antigens are layered onto the serum. For this, into a row of test tubes (according to

the number of antigen dilutions) there is poured 0.2 ml of entire serum into each tube, after which the different dilutions of antigen are carefully added by means of a Pasteur pipette along the wall of the test tube. A distinct border should be evident between the serum and the antigen in the test tube. In the case of a positive reaction there is formed at the border of the liquids, after 30 minutes at room temperature, a dense white or cloudy ring, which is evident in transmitted light.

Setting-up the precipitation reaction in gel proposed by Ouchterlony in 1948. In recent years many authors have modified Ouchterlony's method. The essence of the process of precipitation in gel consists of the fact that the antigen and antibodies diffuse in agar; at the points where they come in contact with one another there are formed several lines of precipitate depending on the structure of the antigen.

The precipitation reaction in gel may be set up in a Petri dish with an even bottom or on a glass 9 X 12 in size (photographic plate), along the edges of which there are glued down, with universal synthetic glue, glass strips 5-10 mm in width and 10-12 mm in thickness. The glass plate should be completely smooth and without scratches. On such a fat-free glass plate there are symmetrically placed 5 uniform (cylindrical 5 mm in diameter, equilateral triangular and rectangular of 5 mm side length) moulds (metallic, hollow): one in the center, four at the periphery at a distance of 10 mm from the edge of the central screen. Then there is poured in a layer

of 1% agar, 3 mm in height. The washed agar is prepared with distilled water, with the addition of an antiseptic (0.02% merthiolate), melting the agar mixture on a water bath and repeatedly filtering this through cotton wool and filter paper until there is obtained a transparent, slightly opalescent solution. When the agar gels on the glass plate the moulds are carefully removed. The pits which have been formed are filled with antigen and antiserum, using Pasteur pipettes: antigen is poured into the central pit and the sera into the peripheral pits, or vice-versa. The glass plate with the agar, antigen and sera is placed in an exsiccator. The results are read out after 1-2 weeks. The reaction proceeds at room temperature or at 37°.

METHODS OF VIROLOGICAL INVESTIGATION

53

The viruses are characterized by two basic characteristics: their small dimensions (from 15 to 500 nm) and their inability to reproduce themselves on artificial nutrient media and extracellularly. Viruses reproduce in vitro only in living cells of tissue cultures, the metabolism of which is provided for by a synthetic nutrient medium. By means of physico-chemical, electron-microscopic and roentgenoscopic methods for studying viruses and by crystallography it has been established in recent years that morphologically viruses have a very complicated submicroscopic structure. They are composed of a protein capsule, which surrounds and protects from disintegration a high-molecular-weight (nucleic) acid. The latter is the infectious agent. The virus capsules, which are composed of protein subunits, are distinguished by their spiral and spherical (cubical) symmetry of structure. Viruses of complicated structure contain, in addition, various component amino acids and lipids.

In 1951 -1953 S. Roegner-Aust et al. found that in virus infections of fishes (carp pox and infectious dropsy of carp) there are present virus particles 40 - 220 nm in size (Figures 4, 5 and 6).

51

Using electron microscopy Fijan et al. (1971) established that the virus of infectious dropsy of carp has a rod-like form with a transverse striation, and that it has a length of about 150 nm and a width of 65 nm.

In many viral infections there are formed special intra-cellular inclusions (intracytoplasmic and intranuclear),

Рис. 4. Элементарные тельца вируса оспы карпа. Электронная микроскопия, увеличение 15 000 (по Регнер-Ауст).

Рис. 5. Элементарные тельца вируса оспы карпа (*а*). Фазово-контрастная микроскопия, увеличение 1350. Окраска по Морозову. Элементарные тельца вируса оспы карпа (*б*). Микроскопия в темном поле, увеличение 1100. Окраска по Морозову.

Figure 4. Elementary bodies of the virus of carp pox. Electron microscopy, magnification X 15,000 (after Roegner-Aust).

Figure 5. Elementary bodies of the virus of carp pox (a). Phase-contrast microscopy, magnification X 1350. Staining according to Morozov.
Elementary bodies of virus of carp pox (b). Dark-field microscopy, magnification X 1100. Staining according to Morozov.

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which are revealed by special histological studies of microscopic sections and impressions of tissues. These inclusions may be of various origin: they are composed either of the virus particles (elementary bodies), or are a product of the cellular reaction to the development of the virus. In

Рис. 6. Эозинофильные внутриклеточные тельца Эпштейна — Пешкова в коже карпа. Обычная световая микроскопия, увеличение 1650. Окраска по Манну — Туревичу (оригинал).

Figure 6. Eosinophilic intracellular bodies of Epshtein-Peshkov in the skin of the carp. Normal light microscopy, magnification X 1650. Staining according to Mann-Turevich (original).

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infectious dropsy of carp , within the intracellular (intracytoplasmic) inclusions of Epshtein-Peshkov (Figure 6) a granular structure may be distinguished by phase-contrast microscopy. This was first noted not long ago by A. A. Avakyan together with the author. It is possible that the intracellular inclusions within the cells of the epithelium of the skin of the carp with infectious dropsy represent colonies of the elementary bodies of the virus.

In order to reveal the localization of the virus, material is selected from various organs. In this case studies are made not only of native but also of fixed material.

Collection of virological material for study

In the collection of the material attention is paid first of all to the superficial pathological changes and then to the results of the pathologico-anatomical dissections. During the dissection it is necessary to strictly observe the 5.
rules of asepsis. The fish is placed on a board, its openings are washed with antiseptic and it is tied down (see Figure 1). The scales are removed with a rasp or knife from the free lateral side of the body of the fish, the pectoral fin is removed and the skin is copiously smeared with a 5% alcoholic solution of iodine or scorched with the flame of a gas burner or blow-torch. The operational instruments should be sterile. For this purpose they are placed in a beaker with alcohol, and before the dissection the scissors, scalpels, anatomical and surgical forceps are passed through the flame of the spirit lamp.

The abdominal cavity is opened along the lateral side by a semi-circular cut (see Figure 2). Before the dissection an incision is made with a scalpel a little above the anus. Into this incision is introduced the blunt end of the scissors, with which the abdominal wall is cut open and turned back. The internal organs may be drawn out only after carefully separating the intestine. Last of all, using sharp-tipped scissors, is dissected the intestine, holding it with surgical forceps so as not to contaminate the material. The skull capsule is cut open or sawed open. The skin, muscles and internal organs are cut out in small pieces and placed into test tubes with a sterile glycerine mixture (see below) or into

empty sterile test tubes for rapid freezing and drying under a vacuum, if the obtained material is not to be studied on the next day. Such material is stored at a temperature of 5°.

Among the liquids for preserving viruses there are widely applied glycerine mixtures of a weakly alkaline pH (pH 7.2-7.4), since the glycerine does not permit the bacterial flora which accidentally gets into the material to develop.

The glycerine mixture is prepared with physiological saline for cold-blooded animals with the addition of sodium biphosphate ($Na_2HPO_4.2H_2O$), in order to neutralize the pH, or to the glycerine there is added a salt buffer, composed of sodium biphosphate and potassium monophosphate (KH_2PO_4) according to the following formulae.

Glycerine mixture with physiological saline:

glycerine . . . . . 50 ml
physiological saline . . . . . 50 ml
sodium biphosphate $^1/_{15}$ N solution (added to mixture to give a pH of 7.4).

The mixture obtained is poured out into test tubes (10-15 ml) and these are then autoclaved at a pressure of 0.5 at. for 20 minutes.

Glycerine-phosphate mixture. First there are prepared the basic solutions of sodium biphosphate and potassium monophosphate:

a) sodium biphosphate ($Na_2HPO_4.2H_2O$) . . . 358.24 gm
distilled water . . . . . 1000 ml
b) potassium monophosphate (KH_2PO_4) . . . 136.16 gm
distilled water . . . . . 1000 ml

The basic phosphate solutions are added to the glycerine in the following proportions:

glycerine (pH not less than 5.5)	500 ml
sodium biphosphate (basic solution)	17.5 ml
potassium monophosphate (basic solution) . .	7.5 ml
distilled water	450 ml

or

glycerine (pH less than 5.5)	500 ml
sodium biphosphate (basic solution)	40 ml
potassium monophosphate (basic solution) . .	10 ml
distilled water	450 ml

The mixture obtained is sterilized in an autoclave at a pressure of 0.5 at. for 20 minutes.

Into 10-15 ml of the glycerine mixture in a test tube is placed not more than 3-5 gm of pathological material. Such material is stored in a refrigerator at a temperature of not more than 5°. For transportation the material may be stored in a thermos (wide-necked) or Dewar flask, into which is placed ice or snow, or into which is poured a temperature-lowering mixture. Dry ice (solid carbon dioxide) may also be used in a Dewar flask without a close-fitting lid (otherwise it might explode!). All of the material contained in the test tubes or flasks is labelled in accordance with the note-book entries, using the form given in the appendix.

Most viruses of man and animals survive for up to several months in 50% glycerine, although their virulent properties are diminished. The virus of infectious dropsy of carp was kept in this way in a refrigerator for up to 6 months

at a temperature of 3-5°; during this period the pathogenicity decreased markedly but the antigenic and immunological properties were retained. Viruses are also preserved for a long time (up to a year) by freezing. In this case, pieces of pathological material are frozen in test tubes, by immersing these in freezing mixtures. The lower the temperature, the more rapidly the material is frozen and the better preserved is the activity of the virus. The virus preserves its virulence best at a temperature of minus 70°, when the freezing of the tissues is not accompanied by crystallization of water.

Formulae for cooling mixtures are presented in Table 1.

Table 1.

Formulae for cooling mixtures (according to S. G. Kolesov)

Name of salt	Proportion by weight of salt per 100 parts snow	Temperature of mixture, °C
Potassium chloride (KCl)	30	-11.0
Calcium chloride ($CaCl_2$)	30	-11.0
Ammonium chloride (NH_4Cl)	25	-15.8
Ammonium nitrate (NH_4NO_3)	60	-17.3
Sodium nitrate ($NaNO_3$)	59	-18.5
Sodium chloride (NaCl)	33	-21.2
Calcium chloride ($CaCl_2 \cdot 6H_2O$)	82	-21.5
Calcium chloride	125	-40.3
Calcium chloride	143	-55.0
Ammonium sulphate ($(NH_4)_2SO_4$)	62	-19.0

A mixture of solid carbon dioxide with alcohol gives up to minus 72°. 58

A better method for long-term storage of viruses consists of the drying of frozen material under a vacuum. The pathological material is ground in the cold in a porcelain mortar with sterile neutral sand. For this purpose river sand is quite suitable but it must be washed in water, then successively in concentrated sulphuric acid, a normal solution of caustic alkali and again in water until it is completely neutralized. The most ideal method for making the suspension is considered to be that in which the tissue is broken up by means of a homogenizer, in which the tissue is rapidly triturated (within 5 minutes) and a homogenous suspension is obtained. The connective tissue of the skin of fishes is difficult to triturate. When using the homogenizer, the container is sterilized by autoclaving, the knives - by alcohol and flaming. The tissue is triturated in a sterile box which is irradiated with BUV-30 bacteriocidal lamps.

The suspension of the pathological material is prepared with physiological saline in a ratio of 1 : 3 or 1 : 5. For better desiccation and preservation of the virus there is added to the suspension an equal volume of normal inactivated blood serum. Before desiccation the prepared suspension is poured out into the sterile vials, which are connected up to the manifold of the drying vacuum apparatus from the trough with the freezing mixture by means of rubber vacuum tubing (sterile), and the material is frozen. In order to trap the moisture, in front of the vacuum pump there is placed a vessel with a water absorber, calcium sulphate (gypsum) in

the form of granules (1-2 cm^3), which has been dried at a temperature of 180-200° for 16-18 hours. The gypsum granules may be used up to 20 times. 1 kg of the gypsum granules absorbs 1 ml of water.*

Drying by the method of sublimation is achieved best at minus temperatures under a vacuum of the order of 0.1 mm of mercury. For laboratory purposes the VN-461 and RVN-20 vacuum pumps are utilized, and these are primed with VM-4 type oil. Between the vacuum pump and the lyophilization apparatus there is positioned a three-way cock in order to prevent the oil from getting into the material. before switching off the electro-motor of the pump the apparatus system is shut off by the three-way cock. The vacuum drying lasts for about one day. After this the vials are sealed under vacuum, by drawing out the neck of the vial on a gas burner or in a hot flame from a spirit lamp, which is made by blowing a strong stream of air through the flame of the spirit lamp.

The period of storage of the vacuum dried material, in a refrigerator at a temperature of 4-5°, is more than a year. For checking the material the vials are opened, distilled water is poured into them and they are left for 1-2 hours in the refrigerator for stabilization of the colloids.

Fixation of material

To preserve the characteristic changes in the tissues the pathological material is fixed. Specific intracellular inclusions and fine cytological changes are best preserved by fixation according to the Duboscq-Brazil method. This method

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*Translator's note. Sic.

of fixation is recommended by the well-known virologist E. I. Turevich for histological studies of the skin and internal organs of diseased fishes.

The Duboscq-Brazil fixative is prepared according to the following formula.

A stock alcoholic solution of picric acid is prepared: 10 gm of picric acid (in powder) and 1500 ml 80% ethyl alcohol.

At the moment of fixation of the material the prepared solution of picric acid is mixed with formalin and glacial acetic acid in the following proportions: 15 ml of the alcoholic solution of picric acid, 6 ml of 40% formalin and 1.5 ml of glacial acetic acid.

The material is fixed well if into the fixative are placed small pieces of tissue no larger than 1 cm^3 in volume per 22 ml of the Duboscq-Brazil mixture. Such pieces are fixed for not less than 48 hours. In a well-capped bottle they may be stored for a long time.

Microscopy of smears, impressions and histological sections

In the collection of patholological material there are prepared fixed preparations for viroscopy*, which do not require complicated histological treatment; these are impressions or smears of the infected tissue on microscope slides. The technique for preparing the smears is simple. Pieces of the infected tissue are cut out, grasped with forceps and applied several times to filter paper to absorb the blood.

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*Translator's note. "virusoskopiya" - an apparently coined term, which has here been translated as "viroscopy" (cf. bacterioscopy).

Then several rows of impressions of the tissue section are made by lightly pressing this onto the microscope slide. In this way are obtained the klatsch-preparations.

For obtaining the smear-preparations a small piece of excised tissue is squashed between two microscope slides. To obtain a thin smear the slides are moved with a sliding motion in various directions. If the preparation is being made from material of liquid consistency (blood, plasma, exudate etc.), then the edge of a cover slip, or of another microscope slide of smaller width, is brought up, at an angle of 45°, to the drop of the material that has been applied onto the microscope slide, the drop is allowed to spread out along the edge and the cover slip is then quickly slid across in the opposite direction.

The microscope slides for the smears and impressions should be thoroughly degreased with "tsettnovskaya"* fluid (a saturated solution of potassium dichromate in concentrated sulphuric acid), in which they are immersed for 2-3 days in a glass vessel. Then the "tsettnovskaya" fluid is poured off, the slides are washed in a stream of tap water and in distilled water. They are dried on filter paper in an inclined position. The clean slides are transferred with forceps into a mixture composed of equal parts of alcohol and ether. The microscope slides should not be thicker than 1.0 - 1.1 mm for microscopic examination by Keller's method or in a dark-field.

Fixation and staining. The selection of the method of fixation of the smear-preparations and the impression-preparations depends on the purpose of the investigation.

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*Translator's note. Direct transliteration.

If the material is unknown to the investigator, then various methods of fixation and staining are applied, since the intracellular inclusions and elementary bodies of different viruses are revealed in a non-uniform manner by different stains. Therefore, for a comprehensive study there are simultaneously prepared as many impression-preparations and smears as is possible. The methods of fixation and staining of the preparations, depending on the aims of the study, are shown in Table 2.

Table 2.

Methods for fixing and staining preparations depending on the aims of the study.

Aim of study	Fixation	Staining
To reveal elementary bodies of viruses in smears and impressions	Dry smears and impressions in Ruge's fluid	According to Morozov
To reveal intracellular inclusions in smears and impressions	Wet smears and impressions in ethyl or methyl alcohol or in a mixture of ethyl alcohol and ether (āā)	According to Muromtsev
To reveal in sections viral elementary bodies, intracellular inclusions of bacteria and protozoans	According to Duboscq-Brazil	According to: Romanovsky-Giemsa; Unna; Mann-Turevich; Lents*.

*Translator's note. Direct transliteration. Possibly Lenz or Lentz.

Viroscopy by Morozov's method has acquired widespread application in practice. It was by this method that the viral elementary bodies of carp pox were successfully revealed. The Morozov method of staining is based on the impregnation with silver nitrate of the minutest particles, which become visible in the normal light microscope, especially with phase-contrast microscopy. Microscopic examination of the preparations made according to the Morozov method on a dark-field permits one to distinguish the viral elementary bodies from cellular protein particles, owing to their peculiar luminescence.

The staining by Morozov's method includes three stages: fixation, mordanting and the staining proper. Correspondingly 3 mixtures are prepared: the fixative (Ruge's fluid) - 2 ml of 40% formalin, 1 ml of glacial acetic acid and 100 ml of distilled water; the mordant - 5 gm tannin, 1 ml crystalline or molten carbolic acid and 100 ml distilled water; the stain (stored in a dark bottle with a ground glass stopper) - 5 gm silver nitrate, 100 ml distilled water and 25% ammonia added carefully drop by drop until the appearance of an opalescence after the appearance of the formation of a soluble brown precipitate.

Thin smears are fixed with Ruge's fluid. They are first immersed vertically for 2-3 minutes in distilled water. Ruge's fluid is poured onto the preparation for 1 minute, this is thoroughly washed off and then the smears are given the mordant treatment with the second mixture for another 1-2 minutes, while being heated over the flame of a burner until vapours appear. After the preparation is washed with distilled water, there is poured onto it the solution of silver nitrate

in a dilution of 1 : 10, and it is slightly warmed over the flame of a burner until the appearance of a dark-brown coloration. After this the preparation is thoroughly washed in tap water and dried in air. Microscopic examination is carried out under the immersion system, initially by the normal light microscope, then with the phase-contrast and, finally, with the dark-field microscope.

Microscopy by the Muromtsev method is generally applied in medicine and veterinary science to reveal the Negri bodies for diagnostic purposes in cases of hydrophobia. In the diagnosis of infectious dropsy of carp or carp pox it was not possible by this method to detect any characteristic specific formations or intracellular inclusions. It is possible that this method may give positive results in the case of other virus diseases of fishes.

For staining by the Muromtsev method fresh smears or impressions in the wet state are fixed by any alcohol (see Table 2) in a vertical trough for 1-2 hours and longer. After the fixative is washed off the preparations are immersed for 5-10 minutes in a diluted (1 : 40) Manson's solution. Then, without rinsing, the preparations are transferred into a 5-10% tannin solution for differentiation to clear the preparation, keeping check of this process microscopically. The preparation should be pale blue. If it turns green, this means that the Manson stain is not of good quality. The differentiation in tannin is stopped by rinsing in water. After being dried with filter paper, the preparation is quickly passed through a mixture, composed of equal parts of absclute alcohol and

ether or absolute alcohol and acetone, and is then mounted in Canada balsam under a cover slip. Smear preparations are examined microscopically under the immersion system and without a cover slip.

As a result of the staining the protoplasm becomes pale blue, the nuclei - dark blue, while various inclusions acquire metachromatic reddish tinges. Bacteria can be detected in the tissues.

To obtain Manson's stain 5-8 gm of chemically pure borax is dissolved in 100 ml boiling distilled water and then to this is added 2 gm methylene blue; the solution is cooled and filtered throuh filter paper. The basic Manson's solution is left to mature in an open flask for up to 6 weeks. Before use the required amount of stain is dissolved in a ratio of 1 : 40. It is best to dissolve the tannin in a thermostat at 6
a temperature of 37° over the course of a day.

Preparation of histological sections. The preparations for histological study are prepared from fixed pieces, embedded in paraffin or celloidin blocks. The latter are not very suitable for virological purposes, since celloidin sections are too thick to reveal intracellular inclusions and viral elementary bodies. Fixed pieces of tissue, 2-3 mm in thickness, are washed in running water and are then gradually dehydrated in a series of alcohols of ascending concentration. For impregnation with paraffin the pieces of tissue are first held in the solvents xylol or benzol. The washing, dehydration and paraffin impregnation of the pieces of tissue is accomplished in the following sequence: washing in running water (in small

gauze bags) - 2 hours, holding in 70% alcohol - 6 hours, holding in 96% alcohol - 15 hours, holding in absolute alcohol - 1 hour, repeated holding in a fresh portion of absolute alcohol - 1 hour, holding in benzol - 1 hour, repeated holding in a fresh portion of benzol - 1 hour, holding in a paraffin paste at a temperature of 37° - 2 hours, first impregnation with paraffin at a temperature of 56° - 1 hour, second impregnation with a fresh portion of paraffin at the same temperature - 1 hour, third impregnation with a fresh portion of paraffin at the same temperature - 1 hour, embedding in paraffin. The paraffin blocks may subsequently be passed through celloidin solutions and so obtaining combined paraffin-celloidin embedding.

In this manner, if the pieces of tissue are begun to be washed, to remove the fixative fluid, at 10 a.m., then the paraffin blocks will be obtained at the end of the following day.

The paraffin blocks are cut on the microtome to a section thickness of 3-5 nm. The paraffin sections are attaced onto microscope slides, which have been smeared with a very thin layer of a mixture composed of equal parts of glycerine and filtered beaten egg white (into this mixture is put a preservative in the form of a small crystal of thymol or camphor). The paraffin sections are carefully smoothed out on the microscope slide in distilled water with slight warming. Then the water is carefully poured off, holding down with filter paper the sections, which are arranged in rows, and centering these on the microscope slide. The attached paraffin

sections are placed in a thermostat for drying at a temperature of 37° for 24 hours. 64

Subsequently the sections are deparaffinized in one of the solvents (xylol, toluol, benzol) and, passing them through a series of alcohols of descending concentration (absolute, 96%, 70%) and water, they are transferred into the desired aqueous solution of stain.

Staining by Unna's method. The preparation, having been passed through xylol, the alcohols and water, is placed in a vertical trough for 15 minutes with a 1% solution of orange yellow (G), it is then rinsed in distilled water and transferred for 40 minutes into a 1% solution of eosin, then again thoroughly washed and stained with a diluted (1 : 5) solution of Unna's polychromic blue for 20 minutes. After rinsing, the stained section is dehydrated, bringing it up through the alcohol series of ascending concentration, and then differentiated in clove oil, dissolved in absolute alcohol (1 : 9), controlling the process under the microscope. From the oil the section is dehydrated by a wash in absolute alcohol and, after passing it through xylol, the preparation is mounted in balsam.

As a result of this staining the erythrocytes acquire a red colour, the protoplasm of the cells - a light-violet, nuclei - violet, inclusions - a dark-pink, elementary bodies and bacteria - a dark-blue colour.

The solution of Unna's polychromic blue is prepared according to the following procedure: 1 gm of methylene blue is dissolved in 100 ml distilled water and to 20 ml of 96% alcohol is added 1 gm of potassium carbonate. The mixture is slowly evaporated on a water bath to 100 ml.

Staining by the Mann - Turevich method. After washing in distilled water the section is immersed for 18 hours in an aqueous solution of the double stain, which is prepared before use. For this are taken 17 ml of a 1% solution of methyl (not methylene!) blue, 23 ml of a 1% aqueous solution of yellowish eosin and 40 ml of distilled water. The stained section is differentiated, checking the progress of this under the microscope, by immersing the section from time to time in an 80% alcoholic solution of tropaeolin "00" until the cytoplasm of the erythrocytes is stained a red colour. Then the section is placed between two leaves of filter paper and carefully wrung out by smoothing this down; the slightly wrung out section is dehydrated for 2-3 minutes by a mixture of phenol and xylol (1 part carbolic acid + 3 parts xylol), then cleared in cedar oil (histological), washed in xylol and mounted in balsam.

The staining by the Mann method, as modified by Turevich, is good for revealing the intra-cytoplasmic inclusions of Epshtein-Peshkov in cases of infectious dropsy of cyprinids, perches and sturgeons. By phase-contrast microscopy it was found that these inclusions are not homogeneous but have a grainy structure.

Unfortunately, methyl blue is in short supply and this limits the application of the Mann - Turevich method.

Staining by the Lents method is a modification of the Mann staining method. In this case methylene blue is utilized in the following sequence. After bringing the section to water, through xylol and the alcohols, it is immersed for

1 minute in a 60% water-alcohol solution of 0.5% eosin, then rinsed in distilled water and immersed for 1 minute in a freshly prepared solution of methylene blue (30 ml of a saturated alcoholic solution of methylene blue and 100 ml of 0.01% caustic potash). The methylene blue is drained off (without rinsing), the preparation is dried off between two leaves of filter paper and the staining of the preparation is differentiated in alkaline alcohol (to 30 ml of absolute alcohol are added 5 drops of a solution of NaOH in 1% absolute alcohol*) until a pink colour is obtained. Then the preparation is rinsed with absolute alcohol and differentiated with acidified alcohol (to 30 ml absolute alcohol is added 1 drop of glacial acetic acid) until a dark blue tinge is attained. The preparation is mounted in balsam, having first been brought through alcohol and xylol.

Microscopic examination. The smears, impressions and histological sections are examined only under the immersion system. The theory of microscopic technique has been excellently presented by M. A. Peshkov (1962). We will pause briefly to consider the technical procedures for maximal exploitation of the optical properities of the microscope according to Keller. The obtaining of a sharp image of the preparation at high magnifications depends on the correct use of the aperture of the iris-diaphragm apparatus of the artificial light source, of the condenser of the microscope, the use of only thin microscope slides and other factors. Our domestic

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*Translator's note. Sic. Pbly. "... of a 1% solution of NaOH in absolute alcohol.

illuminators of the OI-7 and OI-19 types, with an iris diaphragm and a rheostat for regulating the intensity of illumination, are fully adequate for ensuring precise definition. It should be mentioned that the light from the light source is directed only onto the flat mirror of the microscope. First, with a minimal opening of the illuminator diaphragm, the light source is centered onto a white screen, by placing a strip of paper onto the mirror of the microscope. At the same time the image of the lamp filament of the illuminator should be sharp, which is brought about by moving the lamp holder in the illuminator further away or closer. In work with the immersion objective the iris diaphragm of the condenser of the microscope should be completely open, while the field diaphragm of the illuminator should be minimally open.

Sharpness of the image of the preparation should be coincident with a sharp image of the edge of the diaphragm of the illuminator with a X10 ocular and X10 objective. The illuminated circle in the ocular should be in the center of the field of view, which is achieved by turning the mirror of the microscope. After this some immersion oil is applied onto the preparation and into this is carefully lowered the immersion objective. The resolving power of the microscope may be increased if a drop of immersion oil or liquid paraffin is also placed between the microscope slide and the condenser.

There exist methods for finer microscopy in a dark-field and with phase-contrast and "anoptral"* devices for the microscope. Of particular interest is luminescent microscopy

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*Translator's note. "anoptral'nyi" - direct transliteration.

with ultraviolet or blue-violet rays. For this purpose there is used the special ML-2 luminiscent microscope or any biological microscope with the luminescent OI-18 illuminator with a set of light-filters. However in the OI-18 illuminator the SVD-120A mercury quartz lamp is not in all cases suitable, because of an insufficient excitation of luminescence. It is recommended that the spherical DRSh-250 lamp be used. In preparations which are stained with fluorochromes there can rapidly be detected pathological changes in the tissues and organs and there can be revealed individual microbes and viruses. Luminiscent microscopy in a dark-field with an OI-13 condenser gives especially sharp and effective pictures.

At the present time in well equipped laboratories there are electron microscopes, work with which requires special experience, but this does not fall within the scope of the present manual.

Revealing the viral nature of fish diseases 6

In the study of the virus pathogens of fishes experimentation is conducted on fish of the same or closely related species, to which the virus is already adapted. The passaging of the virus should proceed more successfully in the case of the infection of fish of closely related species than in the case of the infection of fish of a distant species. It is especially important moreover that one may reproduce the clinical picture of the disease in a fish of the same or of a closely related species with the same syndrome that is observed under natural conditions. Thus, for example, in the study of

the virus of infectious dropsy of carp we study this virus on the same cultured carp, which, incidentally, is easy to maintain in aquarium conditions.

If the fishes that are subject to the disease (for example, roach, ide, bream, blue bream and others) are difficult to maintain in an aquarium, then one may experimentally infect fish of another species, which adapts easily to aquarium conditions, such as the carp, orfe, crucian carp and some species of exotic aquarium fishes (Macropodus spp.). As our experiment has shown, the virus of infectious dropsy of carp may be adapted in aquarium conditions to the bleak and the orfe, while in natural conditions not only the fishes of another species become infected with infectious dropsy but also fishes of different families (pike-perch, sterlet).

It is probable that with the further development of virological studies of fishes there will be found viruses that are unknown at the present time, which cause diseases of fishes. At the same time it cannot be excluded that newly discovered viruses will turn out to be more specific and less capable of adapting. In any case, with experimental infection it should be borne in mind that after the first inoculation with the virus the infection may proceed asymptomaticly, and therefore in cases of latent infection the virus should be passaged in fish by repeated inoculations.

Experimental infection of fishes and the passaging of a virus

Native material, collected from diseased fish, is tested directly on fish of the same or a closely related species, taken from another body of water where the given disease is not noted or has not previously been noted. The test fish are held in aquaria, troughs or specially set-aside small excavated ponds. The effluent water from these various holding tanks is obligatorily chlorinated. 68

The pathological material is pre-treated. Thus, if it has been stored in a glycerine mixture, it is washed in a sterile manner with physiological saline on a gauze dressing in a glass funnel. The material is weighed in a Petri dish (sterile), cut up with scissors, to it is added a small amount of physiological saline and then it is triturated in a homogenizer or in a porcelain mortar with sand. Then a 10% suspension of the triturated tissue is prepared, by the addition of physiological saline with bouillon or phosphate buffer, so that the suspension will have a particular pH. For example, for the virus of infectious dropsy the suspension should be neutral or weakly alkaline (pH 7.0-7.2).

The phosphate buffer is prepared from a mixture of basic solutions of two salts: $Na_2HPO_4 \cdot 2H_2O$ and KH_2PO_4. The pH of the buffer obtained by mixing these salts is shown in Table 3.

To the homogeneous suspension are added antibiotics (penicillin and streptomycin in doses of 1000 units per 1 ml) and they are left to stand at room temperature for 20-30 minutes.

Table 3.

pH of buffer on mixing different volumes of the salts $Na_2HPO_4 \cdot 2H_2O$ and KH_2PO_4

Таблица 3

pH буфера при смешивании разных объемов солей $Na_2HPO_4 \cdot 2H_2O$ и KH_2PO_4

pH буфера pH of buffer	A Количество раствора солей, мл	
	$Na_2HPO_4 \cdot 2H_2O$ (11,876 г/л) g/l	KH_2PO_4 (9,078 г/л) g/l
8,04	9,5	0,5
7,73	9,0	1,0
7,38	8,0	2,0
7,17	7,0	3,0
6,97	6,0	4,0
6,81	5,0	5,5

A. - Amount of salt solution, ml.

After the contact with the antibiotics the suspension is checked for bacterial sterility by inoculation on MPB, and then the test fish are infected with it. In order to guarantee the bacterial sterility to an even greater degree, a part of this suspension is subsequently centrifuged and filtered through fine-pored filters.

If the tropism of the virus under study is unknown, various methods of infection are tested: rubbing into scarified skin; subcutaneous, intramuscular, intraperitoneal, intracerebral and intracardiac injections, and introduction per os. The presence of the virus in the material depends on the period and stage of the disease, and for this reason a negative result is often obtained. The latter may also be caused by an unfavourable temperature factor of the test environment. The age of the experimental fish is also of

69

considerable significance. Thus, for example, it is more difficult to infect an underyearling carp with infectious dropsy than is the case with carp of older age groups.

In the case of infection by rubbing into scarified skin, a small portion of the skin is cleaned of scales and mucus and is washed with sterile physiological saline. One part of the obtained suspension of pathological material, which is intended for infection by rubbing in, is tested without sedimentation of the triturated tissue; the second part of the suspension, which is intended for infection by injections, is first centrifuged for 10-15 minutes at 3000-3500 r.p.m. The bacteriological sample is taken from the supernatant liquid of the centrifugal product.

Then one part of this liquid is filtered through a fine-pored filter, which holds back bacteria, and this filtrate is used for infecting the fish by various methods, the filtrate is also checked for bacteriological sterility. The remaining part of the supernatant liquid of the centrifugal product is not filtered and is also administered to the fish.

A positive result of an infection of the fish, in the presence of a bacteriological sterility of the filtrate of the studied material, provides grounds for affirming that the studied disease is of viral origin.

The experimental infection of fishes has as its aim not only to reveal the nature of the disease, but also to preserve the characteristics of the initial strain by passaging the infectious agent through a series of fish. Passages of the infectious material in fish stimulate the reproduction of

the virus and strengthen its virulent properties or maintain the pathogenicity. However, at the same time there is a risk of losing the obtained strain of virus in the case of a bacterial contamination of the material and a weakening of its virulence under the influence of unfavourable conditions. Therefore the isolated virus is passaged in fish, paying strict attention to the demands of asepsis in the preparation 70
of the infectious material with its refrigeration under constant bacteriological control. Simultaneously, a viroscopic and histological control is kept on all of the observable changes on the part of the cellular reaction and also of all of the characteristic intracellular inclusions by the usual virological methods of investigation.

Revealing the vectors of a viral infection. The role of vectors of viral infections in fishes in the spread of epizootics has scarcely been studied at all. Meanwhile, the number of possible vectors of fish infections is tremendous, especially among the blood-sucking ectoparasites (crustaceans, leeches), which are found most frequently in the early spring after the period of overwintering.

Employing the virological methods of studying the vectors of viral infections of man and warm-blooded animals, we recommend the administering of a suspension of triturated blood-sucking fish ectoparasites or the application of these ectoparasites to the test fish. The parasites are collected directly from the fish catch, from diseased and superficially healthy fish, in the nidus of the infection, and also from healthy fish from a body of water that is free of the given

disease. These are placed in a test tube or small flask with sterilized tap water or with a glycerine mixture (if the material is intended for prolonged storage), plugged with cotton wool and gauze stoppers.

The suspensions are prepared in the following manner. The collected parasites are repeatedly washed in sterile physiological saline, they are triturated in a mortar with a small amount of neutral sand (sterile) and then MPB is added, with mixing. If the parasites are studied in quantity, for example, the carp louse, then no more than 3 ml of sterile bouillon is taken per 50 specimens. The triturated parasites with the sand and bouillon are sucked up by a pipette into a centrifuge tube and are centrifuged at 2500-3000 r.p.m. for 10-15 minutes.

The supernatant liquid of the centrifugal product is sucked up with a pipette and poured into a test tube. To inactivate any bacterial flora that may accidentally get in there are added 1000 units of penicillin per 1 ml of liquid or it is filtered through bacteria-retaining filters. The obtained liquid is administered to fish in 0.1 ml aliquots. The methods of administration are various, depending on the tropism of the studied virus.

It is somewhat more complicated to study parasites by placing them onto the body of fish, than it is by the method of injection, since it is difficult to attach the parasites to the fish. In any case, some ingenuity on the part of the experimenter is required. On the basis of our own experience, the carp louse Argulus foliaceus and the

leech Piscicola geometra should be applied to the fish in a small volume of water and, as soon as these have attached themselves to the body of the fish, the observations are continued in larger aquaria. These parasites may also be placed, using forceps, directly onto a fish that has been removed from the water. Not more than 5 specimens of the louse or leech should be placed onto each of the test fish, for example onto a yearling carp. With a greater number of parasites the fish becomes restless, it tries to jump out of the aquarium.

Titration of virus. The activity of a virus is determined by titrating the infectious material. The titer of the infectious agent in virology and bacteriology is taken to 71
be that quantity of the pathogen which is capable of causing death in 50% of the experimental animals; this is designated the LD_{50} (the dose for 50% lethality).

For calculating the LD_{50} the statistical method of Rid and Kench is utilized; these authors proposed that no fewer than four animals in each series be taken for testing. The approximate calculation of the LD_{50} by the method of Rid and Kench is presented in Table 4.

In the second and third columns of Table 4 is shown the approximate number of surviving and dead animals on infection with the given dilution of virus; in the fourth column is the number of surviving animals, which is equal to the sum of the survivors at the given and preceeding dilutions; in the fifth, beginning with the highest dilution (10^{-8}), is the number of animals which is equal to the sum of those that

Table 4.

Calculation table by the method of Rid and Mench*

Таблица 4

Таблица расчета по методу Рида и Менча

A. Разведение вирусного материала	B. Число животных в каждом разведении				
	c абсолютные данные		d кумулятивные данные		e смертельность, %
	выжило x	погибло y	выжило x	погибло y	
10^{-1}	0	6	0	25	100
10^{-2}	1	5	1	19	95
10^{-3}	2	4	3	14	82
10^{-4}	2	4	5	10	66
10^{-5}	3	3	8	6	42
10^{-6}	4	2	12	3	20
10^{-7}	6	0	18	1	5
10^{-8}	5	1	23	1	4

A. - Dilution of virus material

B. - Number of animals at each dilution

c - absolute data

d - cummulative data

x - survived

y - dead

e - mortality, %

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succumbed at the given dilution and at all greater dilutions than the given one; in the sixth column is the percentage mortality at each dilution, calculated according to the equation:

$$\text{Percentage mortality} = \frac{100 \times \text{number killed}}{\text{Sum killed and survivors}}$$

For example, the percentage mortality at a dilution of $10^{-4} = \frac{(100 \times 10)}{15} = 66.6$.

The dilutions at which the percentage mortality approaches 50 are called the upper and lower critical doses. The coefficient of proportionality is calculated according to the following equation:

$$K = \frac{\text{Percentage mortality at upper critical dose} - 50}{\text{Percentage mortality at upper critical dose} - \text{percentage mortality at lower critical dose.}}$$

In our example $K = \frac{66 - 50}{66 - 42} = 0.66.$

It has been shown experimentally that in the titration of any material on animals the percentage mortality increases in a direct proportion not to the absolute values of the test doses but to their logarithms. Therefore the LD_{50} is equal to the logarithm of the dilution at the higher critical dose plus the coefficient of proportionality, multiplied by the logarithm of the multiplication factor of the dilution:

$$\lg LD_{50} = \lg 10^{-4} + 0{,}66 \times \lg 10;$$
$$\lg LD_{50} = 4 + 0{,}66 \times 1;$$
$$\lg LD_{50} = 4{,}66.$$

The antilogarithm of this value is equal to 45 710; consequently, in this example the LD_{50} equals 1 : 45 710 (Table 5).

Table 5. Antilogarithms

Таблица 5

Антилогарифмы

Units Единицы	Десятки Tens										Единицы units
	0	1	2	3	4	5	6	7	8	9	
0	1000	1259	1585	1995	2512	3162	3981	5012	6310	7943	0
1	1023	1288	1622	2042	2570	3236	4074	5129	6457	8128	1
2	1047	1318	1660	2089	2630	3311	4169	5248	6607	8313	2
3	1072	1349	1698	2138	2692	3388	4266	5370	6761	8511	3
4	1096	1380	1738	2186	2754	3467	4365	5495	6918	8710	4
5	1122	1413	1778	2239	2818	3548	4467	5623	7080	8913	5
6	1148	1445	1820	2291	2884	3631	4571	5754	7244	9120	6
7	1175	1480	1862	2344	2951	3715	4677	5888	7413	9333	7
8	1202	1514	1905	2399	3020	3802	4786	6026	7586	9550	8
9	1230	1549	1950	2455	3090	3890	4898	6166	7763	9772	9

The titration method should be employed in experimental infections of fishes to elucidate the infectious nature of the disease, since the obtained picture of the lethality in relation to the dilution is characterized by a high reliability of the results obtained.

Centrifugation, filtration and ultrafiltration. The centrifugation and filtration of virological material require considerable attention. If certain conditions are not fulfilled, especially during filtration, this may lead to erroneous conclusions. For virological studies electrical centrifuges are necessary, with a revolution speed of from 2500 to 6000 r.p.m. for sedimenting the coarse tissue and other particles, and with a revolution speed of not less than 30 000 r.p.m. for purifying the viruses from the ballast elements. It is necessary to centrifuge at low temperatures. Among the domestic centrifuges of the first type the TsLR-1 universal laboratory centrifuge with refrigeration is recommended; this centrifuge has two changeable rotors (2500 and 6000 r.p.m.) and a high-speed attachment for 8000 r.p.m. Before centrifugation it is necessary to equilibrate the centrifuge tubes with material in pairs on special Westphal scales, in order to prevent damage to the centrifuge and to avoid a breakdown as a result of the development of an uneven centrifugal force. Among the centrifuges of the second type for work with viruses there is available the domestic UTsP-1 ultracentrifuge with preparative rotors.

For the filtration of virological materials there exist three types of filters: ceramic and asbestos filters

and collodoin membranes ("gradokolovye"*). The ceramic filters of cylindrical form are prepared by the method of Chamberland and Berkefeld.

Chamberland candles have various designations according to the pore size and permeability to bacteria: L_1 with the largest pores, which allow bacteria to pass through; L_2 and L_3 which hold back the larger bacteria; L_5, L_7, L_9 and L_{13} which hold back all bacteria.

Berkefeld candles have the designations: W, N and V in order of pore size. The N candles correspond to Chamberland candles L_2.

The Leningrad Scientific-Research Ceramic Institute produces ceramic filters of various dimensions and degree of porosity; the ~~markings~~ grades of these filters (F_1, F_2, F_3, F_5, F_7, F_{11}) correspond to the ~~markings~~ grades of the French Chamberland filters.

Seitz asbestos filters of the domestic grades F and SF, in the form of laminated discs 35 and 140 mm in diameter, are placed in special metallic Ullengut holders of corresponding diameter (Figure 7). The F filters do not hold back bacteria; the SF filters hold back bacteria and a sterile filtrate is obtained as a result of the filtration. 74

Membrane filters are widely utilized in laboratories. These are prepared from an ether or acetone solution of nitrocellulose; to solutions of nitrocellulose are added defined amounts of methyl alcohol or acetic acid. Membranes are obtained with pores of various gradations, with mean diameters: No. 1 - 350 nm, No. 2 - 500, No. 3 - 700, No. 4 - 900, No. 5 - 1200 nm.

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*Translator's note. Direct transliteration.

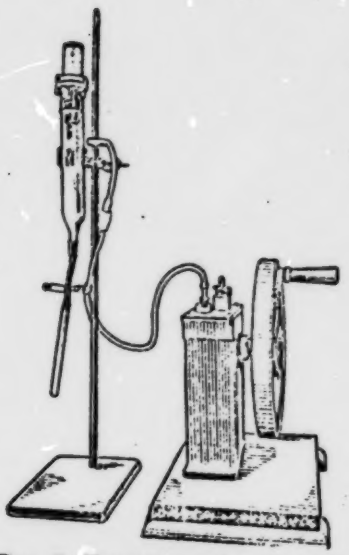

Рис. 7. Металлический держатель Улленгута для фильтрования через фильтры Зейтца и для ультрафильтрования через мембранные фильтры.

Figure 7. Metallic Ullengut holder for filtration through Seitz filters and for ultrafiltration through membrane filters.

Filters No. 1 and 2, which hold back bacteria, give bacteriologically sterile filtrates; Filter No. 3 does not give a complete certainty of obtaining a sterile filtrate, especially in work with an electrical pump, which lowers the pressure drastically.

It is necessary to thoroughly check and treat the ceramic, asbestos and membrane filters before working with these. In the ceramic candles there may be cracks. To detect these, the candles are immersed in water at a pressure of 1 at. and a check is made for the appearance of bubbles of air; a good quality candle does not let through bubbles of air. Filtration candles which have been used are

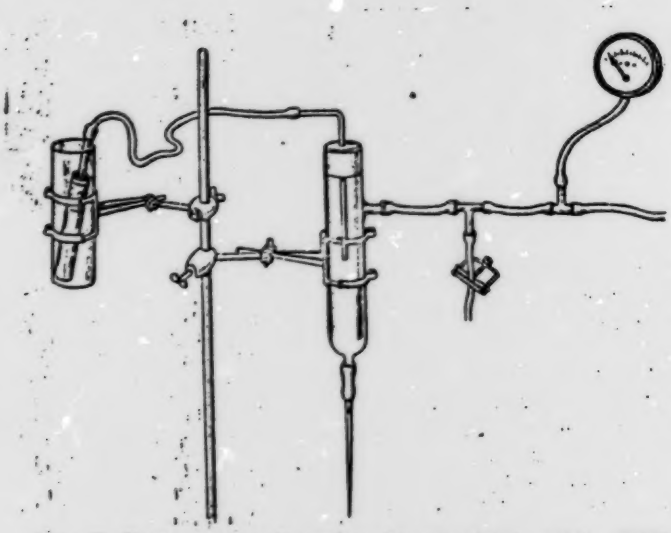

Рис. 8. Смонтированный прибор фильтрования через свечу Беркефельда.

Figure 8. Mounted apparatus for filtration through a Berkefeld candle.

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decontaminated by immersion in a 3% phenol solution for 10-12 hours. After disinfection the candles are washed under pressure in a current of a 1 N solution of hydrochloric acid for two hours, next - with a 2% solution of sodium hydroxide also for two hours and, finally, with tap water until a neutral pH of the wash water is obtained. The washed candles are dried in a thermostat and autoclaved.

Asbestos and membrane filters are not suitable for repeated use. The mounted Chamberland and Berkefeld candles, and also the Seitz asbestos filters are autoclaved together with the receiving vessel, to the end of which a sealed Pasteur pipette is joined by means of a rubber tube (Figure 8).

Before filtration all of the junction points are waxed to avoid the sucking in of air into the receiving vessel under the vacuum. It is very important that the vacuum be

set up gradually and brought up to 25-30 mm of mercury. With a gradual rarefaction of the pressure the pores in the filters do not become clogged. With a high vacuum and rapid rarefaction of the pressure bacteria may pass through into the filtrate.

The probability of the passage of the virus through the pores of the filter depends on the conditions of the filtration. Virus particles are held back by the filter even in the case when they are smaller than the dimensions of the pores. This occurs through adsorption and heterogeneity of the electrical charges on the surface particles of the filter and the virus particles. Of considerable significance in this 76
is the selection of a particular pH of the medium. The permeability of the filter is increased when protein compounds are present in the liquid. Filtration is worse when the material is in physiological saline, than when it is in bouillon. Thus, in the filtration of the virus of infectious dropsy of fishes through the L_3 and L_5 Chamberland candles and the Berkefeld candles it turned out that the best medium was MPB, with a pH of 7.3-7.4. After finishing the filtration the pressure should be equalized with atmospheric, and the filtrate should be decanted from the receiving vessel, through the Pasteur pipette that is attached to it, into sterile test tubes for checking the bacteriological sterility and for subsequent virological studies on the filtrate.

Before working with a membrane filter its upper surface is marked with a pencil. Then it is boiled in distilled water, changing the water several times until there is no smell.

The filtration proceeds successfully only in the case when the suspension is well cleared of the coarse suspended matter by centrifugation or filtration through paper pulp, and also through a No. 5 membrane filter. For the preparation of the paper pulp, filter paper is cut up into small pieces with scissors and placed into a flask, it is covered with distilled water and boiled until a cellulose mass is obtained. The autoclaved cellulose mass is poured out into a Buchner funnel and a dense platelet of 1 cm thickness is obtained, this is then impregnated with sterile MPB under a slight negative pressure (the bouillon which passes through the pulp is poured out of the flask). The supernatant liquid from the centrifugal product of the suspension, containing the virus, is poured into the Buchner funnel with the paper pulp and is filtered under a small pressure. If as a result of the preliminary filtration there is obtained a cloudy filtrate, the filtration is repeated several times through the same paper pulp or through a No. 5 membrane filter until a transparent liquid is obtained.

Treatment of glassware, rubber tubing and stoppers

In virological work with tissue cultures special attention must be paid to the cleanness of the laboratory glassware and rubber objects (test tubes, flasks, pipettes, dishes, tubing, stoppers etc.).

The glassware is treated and washed in the same way as for bacteriological work. The best means for degreasing the glassware is the application of a mixture of OP-10 and

trisodium phosphate or sodium hexametaphosphate. The glassware, which has been washed in hot tap water, is immersed in the detergent solution (10 gm of OP-10 and 15 gm of trisodium phosphate per 10 liters of boiling water) for 20-30 minutes, and is then washed in distilled water.

New rubber stoppers and tubing are boiled for 1-3 hours in a 5% solution of sodium bicarbonate, then washed several times, using a brush, in hot tap water, and again boiled in distilled water (6-7 times, changing the water each time).

Stoppers which have been used previously are autoclaved at a pressure of 1 at. or boiled for 1 hour in a 5% solution of sodium bicarbonate, scrubbed with a brush, rinsed several times with tap water and with distilled water, changing the water three times. The stoppers and tubing are sterilized in an autoclave.

Preparation of nutrient media and salt solutions for tissue cultures

Particular attention is paid to the quality of the distilled and triple-distilled water for the synthetic media solutions. The water is distilled only in a glass vessel with ground glass joints of the still apparatus. For this purpose is recommended a still in the form of a 3-5 liter conical flask with a ground-glass joint for the insertion of a 5-sphere condenser. Into the flask are placed glass capillary tubes, with their ends fused up, in order that the

water boils uniformly. All of the chemical compounds which are included in the solutions and synthetic media are specially purified, the salts are recrystallized and rinsed with alcohol, in which they do not dissolve. Sterile instruments and sterile vessels are used in the work.

Obtaining blood plasma. It is difficult to obtain blood plasma of frogs and fish, since it coagulates rapidly. Coagulation is prevented by heparin. For this purpose, into sterile centrifuge tubes, which have been pre-treated with paraffin and cooled on ice, there is poured 0.5 ml of heparin, in a dilution of 1 : 500, and 5 ml of blood. The tubes with the heparinized blood, cooled with ice, are centrifuged at 2000-2500 r.p.m. for 10 minutes to sediment the formed elements of the blood. Then the plasma is carefully sucked up with a paraffin-treated pipette into paraffin-treated test tubes, in which it is stored for not longer than a week in a refrigerator at a temperature of 4°, tightly closed with a rubber stopper.

The powdered heparin is dissolved in sterile physiological saline for cold-blooded animals and autoclaved at a pressure of 0.6 at. for 10 minutes.

Obtaining fish blood serum from the caudal vein. The fish is first stunned with an electric current, then it is hung on a hook by the gill cover on a Bunsen stand, the body is held by clamps wrapped in gauze and the caudal peduncle is freed of scales and mucus. The skin in the area of the resection of the caudal peduncle is wiped dry and disinfected with alcohol or flamed over the flame of a gas burner. The treated caudal peduncle is cut off with large scissors

below the anal fin from the dorsal side and a test tube is instantly placed under the stream of blood from the caudal vein. The test tube with the blood is set aside for 2-3 hours in an inclined position at room temperature, and is then held for 12-20 hours in a refrigerator. The transparent serum which separates is carefully sucked up by means of the capillary of a Pasteur pipette, without breaking up the clot. For obtaining serum rapidly, the blood is poured into a centrifuge tube, after a few minutes the clot is encircled with a glass rod in a sterile manner and the blood is centrifuged. The serum is poured off into a test tube and, when necessary, inactivated at a temperature of more than 50° for 30-40 minutes. The inactivated or native serum is stored in a sealed ampoule or in a test tube, stoppered with a rubber stopper, in a refrigerator for not more than 5-7 days. Frozen serum, protected from volatilization, may be stored for an extended time.

Nutrient media. Included in the composition of the nutrient media are salt solutions of various formulae (Tyrode, Ringer, Locke - Lewis, Hanks, Earle and others), to which antibiotics are added. Recently, in addition to the salt solutions, an assortment of vitamins and amino-acids is included in the composition of the nutrient media (medium 199).

To the nutrient medium is added embryonic tissue extract. In the classical method for culturing tissue of warm-blooded animals there is used an extract from 7-9-day old chick embryos, which are incubated at a temperature of 37.5 - 38° in a defined humidity. The eggs should be fresh, clean

and unwashed. Before extracting the embryo, the shell is wiped with cotton wool that has been moistened with alcohol. Then, under sterile conditions, the shell at the blunt end is sheared off with sharp-pointed scissors and the embryo is extracted with surgical forceps into Ringer's solution in a Petri dish. The eyes are removed and the remaining tissue is cut up into small pieces with scissors. The cut-up pieces of tissue are rinsed in Ringer's solution, weighed in a tared Petri dish and ground up with a pestle in a porcelain mortar with sand or in a homogenizer with the addition of Tyrode's solution, so as to obtain a 10-30% suspension. The obtained suspension is centrifuged for 10-15 minutes at 2000-2500 r.p.m. The supernatant liquid, the extract, is sucked off with a pipette and stored in a refrigerator at a temperature of 4° for not more than a week. However, K. Wolf (1960) noted that chick embryo extract is contraindicated for the culturing of fish tissues.

For the culturing of tissues of cold-blooded animals a tissue extract from the spleen of healthy fish is prepared in the same manner. The sterility of the extract is checked by bacteriological inoculation. The finely cut-up tissue in a small amount of Tyrode's solution is stored in the frozen state. After a three times repeated freezing and thawing the obtained paste is centrifuged. To 1-2 parts of the supernatant liquid of the centrifugal product there are added 2-3 parts of Tyrode's solution.

Tyrode's solution:

sodium chloride (NaCl) . . . . . (6.5 gm for cold-blooded animals)	8.0 gm
potassium chloride (KCl)	0.2 gm
calcium chloride ($CaCl_2$)	0.2 gm
magnesium chloride ($MgCl_2$)	0.1 gm
sodium monophosphate (NaH_2PO_4)	0.05 gm
sodium bicarbonate	1.0 gm
glucose	1.0 gm
triple distilled water	1000 ml

To the Tyrode solution are added a few drops of a 1% aqueous solution of neutral red till the appearance of a pink tinge of the medium (pH 7.6). The glucose and sodium bicarbonate are dissolved separately and sterilized by filtration through a candle or membrane (or asbestos) filter, while the remaining salt solution is autoclaved at 0.6 at. for 10 minutes. When both solutions cool down, they are mixed together in sterile conditions.

The Locke - Lewis solution was utilized by Grützner in in the culturing of the virus of carp pox and lymphocystosis on epithelial tissues of the embryos of Lebistes and Macropodus. The composition of this solution is:

sodium chloride (NaCl)	7.0 gm
potassium chloride (KCl)	0.42 gm
calcium chloride ($CaCl_2$)	0.25 gm
sodium bicarbonate ($NaHCO_3$)	0.2 gm
glucose	2.5 gm
triple distilled water	900 ml

The sodium bicarbonate and glucose are dissolved separately and sterilized in 100 ml of the triple distilled water by filtration, while the remaining composition of the

solution is autoclaved at a pressure of 0.6 at. for 10 minutes. After cooling, both solutions are mixed in sterile conditions. To the solution there are also added a few drops of a 1% aqueous solution of neutral red.

Ringer's solution (for cold-blooded animals) is used for rinsing pieces of tissue, preparing embryonic and other tissue extracts, diluting viruses etc. Its composition is as follows:

sodium chloride (NaCl)	7.0 gm
potassium chloride (KCl)	0.25 gm
calcium chloride ($CaCl_2$)	0.3 gm
triple distilled water	1000 ml

Hanks' solution is prepared in the form of a concentrated basic salt solution, which is diluted tenfold before use. The salts are dissolved in fresh, triple distilled water in the following composition:

sodium biphosphate ($Na_2HPO_4.2H_2O$) . . .	0.8 gm
or ($Na_2HPO_4.12H_2O$)	1.5 gm
potassium monophosphate (KH_2PO_4)	0.6 gm
potassium chloride (KCl)	4.0 gm
magnesium sulphate ($MgSO_4.7H_2O$)	2.0 gm
glucose	10.0 gm
sodium chloride (NaCl)	80.0 gm
triple distilled water	900 ml
calcium chloride ($CaCl_2.6H_2O$)	1.4 gm

(The last is dissolved separately in 100 ml of triple distilled water.)

In order to avoid the formation of a precipitate, the solution of calcium chloride is slowly added to the solution of the first salts after these have been dissolved. The obtained solution is filtered through sterile filter paper

and 4 ml of chloroform are added as a preservative to each 1 liter of solution. In this form the solution is stored at a temperature of 4° for an unlimited time in bottles with tightly fitting ground glass stoppers (rubber stoppers are unsuitable, since they are subject to the action of the chloroform vapour).

The solution for use is diluted from the basic solution in the following manner: to 100 ml of the basic solution are added 900 ml of triple distilled water and 2 ml of 1% phenol red, so that a change in the pH can be detected. The diluted solution is autoclaved at a pressure of 0.6 at. for 10 minutes. After cooling, 26 ml of an autoclaved 1.4% solution of sodium bicarbonate are added, in sterile conditions, per 1 liter of the solution for use. By passing through the solution a mixture of 50% air and 50% CO_2 a pH of 7.4 - 7.6 is attained. The colour of the solution in the presence of phenol red should be orange-red. The solution is poured out into sterile ampoules or penicillin vials with the help of a siphon and double rubber bulb. The ampoules are sealed, while the vials are closed with sterile rubber stoppers. The solution is stored at a temperature of 4° in a refrigerator for not more than 2 weeks.

The 1% solution of phenol red is prepared with triple distilled water, applying heating to the boiling point several times.

Kholtfreter's * solution (according to J. Paul, 1965):

sodium chloride ($NaCl$)	3.5 gm
potassium chloride (KCl)	0.05 gm
calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.1 gm
sodium bicarbonate ($NaHCO_3$)	0.2 gm
double distilled water	1000 ml

This is sterilized by filtration through an SF asbestos filter.

The solution of Dulbecco and Vogt, as modified by Wolf, is prepared according to the following formula:

sodium chloride ($NaCl$)	8.0 gm
potassium chloride (KCl)	0.2 gm
sodium biphosphate (Na_2HPO_4)	1.065 gm
potassium monophosphate (KH_2PO_4)	0.249 gm
triple distilled water	800 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

calcium chloride ($CaCl_2$)	0.1 gm
triple distilled water	100 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

magnesium chloride ($MgCl_2$)	0.1 gm
triple distilled water	100 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

After cooling the prepared solutions are mixed together in sterile conditions. 8

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*Translator's note. Direct transliteration.

Earle's solution is prepared according to the formula:

sodium chloride (NaCl)	6.8 gm
potassium chloride (KCl)	0.4 gm
magnesium sulphate ($MgSO_4$)	0.1 gm
sodium monophosphate (NaH_2PO_4)	0.125 gm
triple distilled water	800 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

calcium chloride ($CaCl_2$)	0.2 gm
triple distilled water	100 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

sodium bicarbonate ($NaHCO_3$)	2.2 gm
(or for Wolf's nutrient medium)	1.1 gm
dextrose (glucose)	1.0 gm
triple distilled water	100 ml

This is autoclaved separately at a pressure of 0.6 at. for 10 minutes.

After cooling all of the prepared solutions are mixed together in sterile conditions.

Wolf's nutrient growth medium (1960). This is prepared according to the following formula:

human placental blood serum	10 ml
chicken egg (ultrafiltrate)	5 gm
medium 199	30 ml
lactalbumin hydrolysate	0.5 gm
Earle's solution	45 ml
Hanks' solution	10 ml
penicillin	40 000 units
streptomycin	40 000 units
nystatin	5000 units

Wolf states that this nutrient medium gives positive results in the culturing of tissue of various cold-blooded animals. In the experiments of Wolf and Dunbar (1957) positive results were obtained with the addition of chicken and rabbit blood serum to this medium. Carp serum supported the growth of trout kidney tissue.

For the Wolf medium a fresh chicken egg is cut open in a sterile manner. The white and yolk are poured into a sterile homogenizer flask and a homogenous emulsion is obtained. The latter is pre-filtered and then filtered again through a Seitz SF asbestos filter, gradually setting up the vacuum in the receiving vessel.

Medium 199 represents a complex solution of 60 components: various amino-acids, vitamins, lipoid sources, nucleic acid components, carbohydrates and salts. It may be obtained in the prepared form. The required amount of the medium is collected by means of a syringe through a rubber stopper. In order to provide access for sterile air into the flask, into the stopper of the flask is inserted a second thick syringe needle, fitted with a rubber tube and cotton wool plug (air filter).

The weighed aliquot of lactalbumin hydrolysate is dissolved in the Earle or Hanks solution. This mixture is filtered through a No. 2 membrane filter or an SF asbestos filter. The antibiotics are dissolved in the Earle or Hanks solution. If, for example, the vial with the crystalline sodium salt of penicillin contains 100 000 units for injection, then by means of a syringe 2.5 ml of the Earle or Hanks

solution are introduced, so that the penicillin concentration would comprise 40 000 units in 1 ml per 100 ml of the Wolf medium.

Osadchaya's nutrient growth medium (1965):

"kompleksamin"*	0.025 gm
hyaluronidase, 2% solution	0.1 mg
calf blood serum	10.0 ml
Hanks' solution	up to 100 ml
penicillin and streptomycin	200 units of each per 10 ml.

The medium has a pH of 7.1 - 7.2. Bovine amniotic fluid may be added. The "kompleksamin" contains 16 amino-acids; it was obtained from the Laboratory of the Ukrainian Research Institute of the Meat and Milk Industry from carp tissue.

Goncharov's nutrient growth medium:

Kholtfreter's solution	50 ml
medium 199	30 ml
calf blood serum	20 ml
lactalbumin hydrolysate	0.5 gm
penicillin and streptomycin	400 units of each per 1 ml

Disaggregating media. To obtain monolayer cell cultures the tissues of the fish organs are first disaggregated. For this purpose the tissues of the fish are 81
treated with Versen's** solution or solutions of 0.25% trypsin and also 0.1-0.2% pancreatin in Hanks' solution. The proteolytic activity of the trypsin and pancreatin is verified on the gelatin on an exposed film.

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* and ** Translator's note. Direct transliterations.

All of the nutrient and disaggregating media are sterilized by filtration through SF asbestos filters.

Methods of obtaining tissue cultures and infecting these with viral material

Success in growing tissue cultures depends primarily on a good organization of the laboratory work. The nutrient media, solutions and glassware should be prepared under the best possible aseptic conditions.

The thorough chemical treatment of the laboratory ware, the purity of the chemical reagents and the quality of the distilled water are of considerable significance, as is the application of antibiotics (penicillin and streptomycin), which prevent the contamination of the cultures by bacteria. One should make use of immaculately purified crystalline streptomycin (the chloro-calcium complex for endolumbar use) and penicillin (sodium salt).

At the present time there are distinguished two basic groups of methods for culturing tissues: those allowing survival of the cells of explants and those allowing growth of the cells of disaggregated tissues. To the first group belong the classical methods of Carrel (1912), Maksimov (1925), the Maitlands (1928) and others. To the second group belongs the method of Dulbecco and Vogt (1954). This method was first used by Grützner in 1958 for obtaining cell cultures from fish tissues, while in 1960 K. Wolf with coworkers from his laboratory (USA) applied a modification of this method for obtaining monolayer cell cultures of fish tissues. The latter method

has proved to be a good procedure and has been included in ichthyological practice for studying the virus diseases of fishes.

Culturing cells of explants for survival. Maksimov's method is based on the culturing of tissue on a solid medium, on coagulated plasma in a moist chamber (a special microscope slide with a concavity (Figure 9, a). If a culture is to be made of a tissue that is not free of bacterial flora, then this tissue is fragmented with scissors in a Petri dish and pre-treated with antibiotics, at a dose of 1000 units of penicillin and 1000 units of streptomycin per 1 ml of suspension in Tyrode's solution. The period of contact of the antibiotics with the tissue under study is established experimentally by bacteriological controls. 8

To obtain the culture a drop of plasma is applied onto a cover glass, then into the plasma is placed a small piece of the treated tissue and the tissue extract is added; as soon as the plasma coagulates on combination with the latter, there is applied onto the surface of the solid phase a drop of the liquid phase mixture, composed of serum, Tyrode's solution and antibiotics. The ratio of the parts of the mixture is established experimentally.

The obtained tissue culture on the cover glass is fastened by a drop of Tyrode's solution to a mica flake and positioned over the concavity on a Maksimov microscope slide; with the aid of forceps the edges of the mica flake are are circled with sterile molten paraffin. Fish tissue cultures are raised at room temperature. The zone of growth of the tissue fragment is detected under low microscopic magnification.

а a *б* b

Рис. 9. Стекло Максимова (*а*). Чашка Карреля (*б*).

Figure 9. Maksimov microscope slide (a). Carrel flask (b).

Carrel's method is based on culturing the tissue on a solid medium, on coagulated plasma, but in closed flasks with a sloping neck (Figure 9, b). Carrel flasks are more convenient in practice than the Maksimov microscope slides, since they permit the nutrient medium to be refreshed and by this the period of culturing of the tissue can be lengthened, and they also give a larger amount of material. The principle of culturing by the Carrel method is the same as that of the Maksimov method. After the introduction of the nutrient medium and pieces of tissue into the Carrel flask the neck is tightly plugged with a sterile rubber stopper.

Using the classical methods of Maksimov and Carrel for culturing tissues, Grützner first demonstrated, in 1956, that carp pox and lymphocystosis in the flounder were of viral origin.

We will consider only Grützner's experiments with the pox. Serving as the initial material were the variolar formations on the skin of the carp, which were frozen at a temperature of minus 70°. The variolar material was treated on the day prior to infecting the tissue culture. To one part,

by weight, of the frozen material, which was triturated in a porcelain mortar, there were added 4 parts, by weight, of the Locke - Lewis solution. The suspension obtained was centrifuged for 15 minutes at 2500 r.p.m. To the supernatant liquid of the centrifugal product there were added 100 μg of streptomycin per 1 ml, since the variolar material from the skin was not free of bacterial flora. The contact of the antibiotic with the material lasted for 6 hours at room temperature, after which the material was subjected to bacteriological control for sterility. The obtained suspension of the pox virus was frozen at a temperature of minus 70° and stored until its bacterial sterility was elucidated. If the suspension was found to be sterile, then the tissue cultures were infected with it.

To obtain as homogeneous as possible conditions for the experiment, the transplants for infection were utilized after they had been cultured for 48 hours on the artificial medium. Tissue which produced an abundant growth of cells was generally employed. The embryonic epithelium of the guppy and Macropodus served as such tissue, since the embryonic tissue is better for adaptation, because, as was pointed out by Grützner, the defensive antibodies have not yet been formed in the organism.

As a rule, the original fragment of epithelium with its zone of cell growth on the artificial medium was divided into 2 equal portions before infection. One portion of the explant served as the control, while the second was infected by contact with the virus suspension for 1 hour. Because of

a certain toxic effect of antibiotics on the tissue cultures, control cultures were set up with the addition of 100 μg of streptomycin per 1 ml in the Locke - Lewis solution (as was added to the infective material). After such treatment the infected and control explants were transferred into a medium composed of one drop of embryonic extract and one drop of heparinized chicken plasma. The cultures were made on Maksimov slides or in Carrel flasks. The tissue cultures were transplanted into fresh medium after 3-4 days. Explants with a large zone of growth were first divided into halves. A cytopathic effect was detected in the form of cytoplasmic and nuclear changes only after 14-28 days.

Explants with observed changes were fixed with 2% formalin in Locke - Lewis solution for two hours, then thoroughly rinsed in tap water and on the same day stained by the Giemsa method (24 hours) at room temperature (1 drop of Giemsa stain per 2 ml of freshly prepared distilled water). The preparation was differentiated in tap water, dehydrated through an acetone series, mounted in Canada balsam and examined microscopically to reveal the cytopathic effect.

Experiments on 120 cultures showed that the agent of carp pox affected the cells of the explants. The degree of proliferation was higher than in the control after several passages. In the infected cells cytoplasmic and nuclear changes were observed in the form of a greater polymorphism of the nuclei and a greater vacuolization of the cytoplasm on the 13th - 35th day after infection. Giant cells developed in the infected explant always closer to the edge of the

growing portion. These were 2-4 times larger in size than the normal epithelial cells in the control. However the specificity of the inclusion bodies formed in the cells of the infected tissue cultures has not yet been demonstrated.

Obtaining monolayer transplantable cell cultures. Lying at the basis of this method is the work of Dulbecco and Vogt (1954). It was first employed for fish tissues in Germany by Grützner (1958) for obtaining monolayer cell cultures of liver and kidney of the tench. In 1960 K. Wolf and his coworkers modified the method of culturing and were subsequently able to obtain eurythermic transplantable lines of cells from the gonads of the rainbow trout RTG (1962) and from the tongue of a frog FG (1964). Since that time monolayer cell cultures have also been obtained by other investigators (Table 6).

To obtain the monolayer cell cultures K. Wolf and his coworkers, following the method of Bodian (1956), first disaggregated the tissue with trypsin, For this purpose they dissolved the trypsin in the phosphate buffer of Dulbecco and Vogt, in the following ratio to the nutrient medium:

Modified Dulbecco and Vogt solution . . .	37.5 ml
solution of Wolf's nutrient medium . . .	10.0 ml
0.5% solution of trypsin in Dulbecco's solution	50.0 ml
human placental blood serum	2.5 ml
penicillin and streptomycin . . .	20 000 units of each

Tissue for trypsinization was taken by K. Wolf in a sterile manner in a ratio of 1 : 10. Before trypsinization small pieces of trout tissue were held in the nutrient medium

Table 6.

Isolated cell culture	Line	Author
Liver and kidney of tench	-	Grützner, 1958
Fins of various marine fishes	-	Klem et al., 1961
Gonads of rainbow trout	RTG-1	K. Wolf et al., 1962
Tongue of frog	FG	K. Wolf et al., 1964
Kidneys, swim bladder, embryos of trout	-	Ensen, 1963
Gonads, swim bladder of tench	-	Pfitzner and Grützner, 1964
Kidney of carp	-	Osadchaya, 1964
" " "	-	Tomasec and Fijan, 1965
Embryos of frogs	WERP	Granov et al., 1965
Kidneys of tadpoles	KFRS	Granov et al., 1965
Fins of "golopuzok"* (a marine fish)	FHM	Gravell and Mal'sberger, 1965
Immature gonads and various organs of tench, trout and embryos of guppy	-	Pfitzner, 1965
Fry of sunfish	BF-2	Zwillenberg and K. Wolf, 1968
Fins of "gemulid"*	GF	Klem et al., 1965
Kidneys of bream	-	Goncharov and Zubkova, 1970
Gonads of carp	-	Fijan et al., 1971

* Translator's note. Directly transliterated. I was unable to find a translation for these Russian names of fishes.

Please note also that the authors' names that I was unable to check out (Klem, Ensen, Granov, Gravell and Mal'sberger) have also been directly transliterated.

for 24 hours at a temperature of 4°, while the tissues from other fishes were held in a vessel on ice. The disaggregation of the tissue was carried out in an Erlenmeyer flask with the help of an electromagnetic stirrer at a temperature of 4-6°. The whole process of disintegration of the tissue lasted for from 3 to 20 hours (depending on the sample). The first portion of disaggregation was generally poured off and discarded. Then the trypsin solution was again added and the flask placed on the electromagnetic stirrer. The best results of trypsinization were obtained when this process lasted no more than 20 hours. The practical yield comprised 10^5 - 10^6 cells in 1 ml during a night of treatment.

The obtained cell suspension was sedimented by centrifugation in the cold and on average there was obtained 0.1 of the initial volume of tissue taken for trypsinization. The cells were washed once in a cold salt solution which contained from 2 to 5% blood serum, and were then suspended in the nutrient medium mentioned above. The suspension was diluted in a ratio of 1 : 400 - 1 : 600. At such a dilution the number of cells comprised $1 \cdot 10^5$ - $3 \cdot 10^5$, with the pH of the medium being 7.2 - 7.4. The cells were incubated at a temperature of 12.5°, though it is better to incubate at 19°.

In this way, from an initial culture in January 1960, K. Wolf and Quimby (1962) created a line of cells, RFG-2, from several gonads of sexually immature rainbow trout (yearlings); the formation of ovaries could be distinguished macroscopically in the gonads. The original cell cultures were obtained after dispersion of the gonads by trypsinization,

as was mentioned above, and the cultures were left in test tubes in a static state. In the monolayer there readily developed a mixture of epithelial-like and fibroblast-like cells. Initially in the subcultures the cells developed during the course of the second and third weeks. During the course of 21 months the cell line provided subcultures more than 48 times. Transplantations were carried out at an interval of 1-2 weeks, depending on the sensitivity of the parental cells, the temperature of incubation and the pH of the medium. The medium was not renewed between transplantations. K. Wolf and Quimby utilized Eagle's medium, NCTS-109 and other media for human Hela cancer cell lines, with the addition of 10-15% foetal calf serum.

The RTG-2 cell line withstands a prolonged incubation period without feeding. At a temperature of 19° the cultures did not lose their viability and produced mitoses even after 5 months, but they utilized glucose and the medium became slightly acid. They also survived when the cultures were stored at a temperature of 4° for 9 months. Taking into consideration these characteristics of the RTG-2 cell line, the interval between transplantations may be increased to two months. Subcultures withstand dispersion of the cell layer with 0.25% trypsin, but it is better to use Versen's solution for 10 minutes in the cold after direct neutralization of the old medium. The morphology of the RTG-2 cells is influenced by the medium, the age of the culture and their position in the layer, but in most cases the cells have a fibroblast-like form. After dispersion by Versen's solution the cells become

spherical in form, with a diameter of 5.2 - 23.4 nm, averaging 5.6 nm. The obtained RTG-2 cell line, according to K. Wolf and Quimby, possesses a high sensitivity to the virus of infectious pancreatic necrosis of trout, and, according to Fijan (1971), - to the virus of infectious dropsy of carp.

Fryer et al. (1965), who studied the conditions of culturing tissues and cells of salmonid embryos, showed that the successful growth of monolayer primary cell cultures depends on the choice of the enzyme for disaggregating the tissue, on its concentration and on the quantity of living parental cells taken for culturing, and on the composition of the nutrient medium. According to their data, the best enzyme for freeing the cells from the tissues of salmonids is trypsin at a 0.25% concentration in a balanced Hanks' solution (pH 7.4). They recommend that the first disaggregation of the tissue be carried out for 15 minutes at 15°, after which the first portion of the suspension of freed cells is discarded, while the disaggregation is continued on the remaining pieces of tissue, pausing every 30 minutes to draw off the cell suspension and replace the trypsin solution with fresh solution. These authors centriguged the suspension of free cells for 20 minutes at 600 r.p.m., and after removing the trypsin solution they poured nutrient medium onto the sediment. This nutrient medium was composed of Eagle's medium with 20% gamma-globulin-free bovine serum to which were added the essential and seven non-essential amino acids and also inositol.

The dilution of the cell suspension in the nutrient medium was brought to $15 \cdot 10^5$ cells per 1 ml. Then the cells

were cultivated in special 4-sided vials and left in a stationary slanted position for 48 hours in a thermostat at 18 and 23°. At the end of this period the nutrient medium was changed and incubation was renewed. The lag-phase of the growth of the culture lasts for approximately 3½ days, and then the logarithmic phase of growth commences. The monolayer of the cell culture is formed in the course of two weeks. Subsequently, subcultures were obtained from the cultured cells in the monolayer, which was achieved with the aid of a 0.25% solution of trypsin, that separated the cells from the surface of the glass, and a washing away of the trypsin by centrifugation. The sediment of cells was resuspended in nutrient medium at a cell concentration of up to $6 \cdot 10^5$ per 1 ml. The nutrient medium in the culture was refreshed at 2 - 3 week intervals.

In obtaining the primary monolayer cell cultures from the embryos of salmonids, according to Fryer, there may be variations in the technique of production depending on the object selected.

In the Soviet Union in the Ukraine E. F. Osadchaya (1964, 1965) first obtained monolayer cell cultures from the kidneys and gonads of carp. She found that it was most expedient to treat the kidney tissue for disaggregation with a 0.2% solution of pancreatin for 30-60 minutes at 16-18° or for 20 hours at 4° under the condition of the addition to the pancreatin solution of 2.5% calf blood serum. As the nutrient medium for cell growth E. F. Osadchaya recommends a medium, the composition of which includes "kompleksamin". According

to the data of E. F. Osadchaya, the freed kidney cells of the carp at a concentration of $5\text{-}8 \cdot 10^5$ per 1 ml of nutrient medium begin to grow and to adhere to the glass surface of the test tube within the first 24 hours and frequently retain their viability for 12-20 days at a temperature of 16-20°.

An attempt by the present author (Goncharov et al., 1970) to obtain monolayer cell cultures from the kidneys of the bream (sexually immature) by various methods showed that Fryer's method gives better results in culturing the cells of this species of fish, though in conditions of the utilization of another set of media for disaggregating the tissue and for growing the cells.

Before removing the kidney the fish should be killed by an electric current (applying one electrode to the head and the other to the caudal peduncle for a few seconds) and then exsanguinated, as indicated above. The kidneys, which are removed in a sterile fashion, are transferred into a Petri dish with a disaggregating medium of the following composition:

pancreatin	0.2 gm
Kholtfreter's or Hanks' solution	97.5 ml
calf blood serum	2.5ml
penicillin and streptomycin	400 units of each per 1 ml.

The medium should be alkaline, with a pH of 7.2.

The kidneys are cut up into small pieces with scissors and transferred into an Erlenmeyer flask of 100-150 ml capacity. The small pieces of kidney are flooded with fresh pancreatin solution in a ratio of approximately 1 : 10, so that the layer of liquid in the flask covers the fragments to a

depth of 10-15 mm, and the flask is placed on an electromagnetic stirrer at room temperature. The obtained suspension of freed cells is poured off and fresh solution is added every 15 minutes. The first portion with elements of the blood is discarded, while the following ones are poured off or carefully sucked up with a pipette into centrifuge tubes and centrifuged at a small number of revolutions (not more than 500 r.p.m.) for 10-15 minutes to sediment the cells. Then the supernatant liquid with the pancreatin is poured off and the sediment is resuspended in the nutrient growth medium (Goncharov's medium). The obtained cell suspension is diluted with the nutrient growth medium to a concentration of $15 \cdot 10^5$ cells in 1 ml, following Fryer's recommendation. The number of cells is counted in a Goryaev chamber. Then this suspension is poured out in 1 ml aliquots into test tubes that are flatenned at their lower ends, which are placed onto special wooden trays in an inclined position for 48 hours in a thermostat at 20-25°. During this time the cells settle and adhere closely to the glass surface of the test tube. On the third day the nutrient medium is refreshed, in the course of 1-2 weeks there is formed the monolayered primary cell culture in the form of a delicate film on the surface of the glass. Then the nutrient medium is sucked up from the test tube with the culture, it is replaced for a few minutes by the above-mentioned disaggregating solution of pancreatin and, with the aid of a glass rod with a rubber tip, the culture of cells is removed from the surface of the glass. The obtained cell suspension in the pancreatin solution is freed from the latter

by centrifugation, some nutrient growth medium is poured onto the sediment of cells, a count is made in a Goryaev chamber of the number of cells in 1 ml of medium and the suspension is poured out into sterile test tubes with the growth medium in an amount such that the concentration of the suspension of original cells in the subcultures is $6 \cdot 10^5$ in 1 ml. The subcultures are transplanted as they grow.

In Figure 10 is shown a photomicrograph, using the phase contrast method, of a 5-day living, unstained, monolayer culture of bream kidney cells.

Рис. 10. 5-суточная однослойная культура клеток почек леща. Фазовый контраст. Увеличение 550.

Figure 10. 5-day monolayer culture of kidney cells of bream. Phase contrast. Magnification X 550.

Infection of cell cultures with viral material.

As an example of the application of monolayer cell cultures for virological purposes we will cite only the last, recently

published study by Fijan and his coauthors (1971), since several other studies on the isolation and culturing of the viruses of fish diseases are methodologically similar.

It should be noted that during the last ten years monolayer primary cell cultures, especially established cell lines, have been widely employed in virological studies of fish diseases. Thus, since the studies of E. F. Osadchaya (1965, 1970), of I. Tomasec and N. Fijan (1965) with primary monolayer cell cultures, and especially since the studies of N. Fijan and his coauthors (1971) on the cell lines FHM, BB, B7-2 and RTG-2, the debated question concerning the viral nature of the pathogen of infectious dropsy of carp may be considered to have now been resolved positively.

Before infecting the cell cultures, the pathological material, which was frozen at minus 70°, is weighed, thawed out and homogenized before testing in a mortar using a pestle in Hanks' solution at a ratio of 1 : 10. The suspension obtained is centriguged at 2000 r.p.m. for 10 minutes at room temperature, the supernatant liquid is filtered through a fine-pore filter of 450 nm pore-size (corresponding to our domestic membrane filters No. 1 and 2, and the SF asbestos filter). A tenfold dilution of the obtained filtrate is used to infect the primary cell cultures (series of test tubes), for example cultures of the FHM cell line and others at an age of 1-4 days, and these are incubated at room temperature for 1 hour. For the isolation of the virus of infectious dropsy of carp E. F. Osadchaya infected primary cell cultures of gonads with a well formed monolayer at 24-26° on the 5th-7th

day. Infection of the cultures was carried out at 20-22°, after first pouring the nutrient medium off it, by introducing 0.2 ml aliquots of the obtained filtrate and incubating at room temperature for 30 minutes.

In the experiments of Fijan et al. the start of the development of a cytopathic effect (CPE) occurred approximately after 40 hours at a temperature of 10° with a filtrate dilution of 10^{-1}. The cells in the monolayer of the culture rounded up and detached from the glass. At greater dilutions of the filtrate (10^{-2} and 10^{-3}) such changes develop, become focused and progress with time. After 8 days all of the cells in the cultures with a CPE separate from the glass and are lysed.

A quantitative count of the virus of infectious dropsy of carp in an infected FHM cell culture, which was incubated at room temperature (17°-22°), was carried out on the 10th day by determining the lethal dose, LD_{50}, to the cell culture by the statistical method according to Rid and Mench.

A content with a high concentration of virus evokes CPE in a monolayer cell culture in the form of degeneration and destruction of the cells in the whole layer. In cultures with focal CPE there are found nuclear changes in the cells, there occurs a granulation of the chromatin and a thickening or lysis of the nuclear membrane, and also a cytoplasmic degeneration. This is clearly evident on the photomicrograph of the preparation, stained by the May-Grünwald method (Figure 11, a, b).

Рис. 11. Цитоплазматическая дегенерация, грануляция и краевое скопление ядерного хроматина клеток, зараженных вирусом краснухи карпа (*а*). Увеличение 1430. Фокальный цитопатический эффект (*б*). Увеличение 230. Окраска по Май—Грюнвальду (по Фиджену с соавторами).

Figure 11. Cytoplasmic degeneration, granulation and regional accumulation of nuclear chromatin of cells infected with the virus of infectious dropsy of carp (a). Magnification X 1430.
Focal cytopathic effect (b). Magnification X 230. Stained by the May - Grünwald method.
(according to Fijan and coauthors).

The CPE is clearly evident under low magnification (X 200) of the microscope through the glass on the back side of a flat vial or the flattened part of the test tube. A thin cytological preparation for observation under high microscopic

magnification, under immersion, is obtained either from the suspension of a washing of the culture layer or from a layer of cells that is formed on cover glasses which are placed into the culture tubes.

Serological methods of study

Up to the present time serological methods for studying viral diseases of fishes have not been included in ichthyopathological practice, although we (Goncharov, 1949) were able to show, in the course of a study on the etiology of infectious dropsy of fishes, that serological diagnosis of this disease was quite possible by establishing the presence of antiviral antibodies. Thus, studies by virologists (Roberts, 1941; Sergiev, Ryazantseva and Demina, 1945) have helped to diagnose infectious dropsy of fishes as a viral disease, by means of the reaction of agglutination of bacteria that are loaded with virus (ABV reaction). Then, in Poland, M. Dubowicz and B. Kocylowski (1956) successfully employed the haemagglutination reaction for the diagnosis of infectious dropsy. Up to the present time nobody has yet applied other serological reactions (neutralization and complement fixation) with respect to viral diseases of fishes.

The bacterial-viral agglutination reaction (ABV reaction). The essence of this reaction consists of the fact that antiviral serum together with bacteria may agglutinate viruses that are adsorbed, under particular conditions, on the surface of bacterial cells.

The investigators who developed this diagnostic method for human viral diseases selected as the adsorbents such bacterial cultures which were noted for their agglutinative capacity, which at the same time did not agglutinate spontaneously and, mainly, which adsorbed the virus. Roberts (1941) found a suitable culture for this purpose: of Serratia marcesens, which adsorbed the virus of encephalitis. Ryazantseva and Demina (1945) utilized a bacterial culture of Ebertella typhi, which adsorbed the viruses of measles and influenza. As the adsorbent we selected one of the laboratory strains of Bact. septicaemia ranarum, which adsorbed the virus of infectious dropsy. If this species of bacterium is not available, one may select any other species of the gram-negative bacilli of small length (1.0 - 1.2 nm) that are capable of adsorbing the virus.

The ABV reaction requires that certain standard conditions be observed: all of the glassware and all pipettes must be chemically clean and sterile; for the dilution of the sera it is necessary to use not buffer solutions but a physiological solution of sodium chloride; the suspension of the virus should be clarified by centrifugation; the reaction must be set up in sterile conditions; the completeness of the adsorption depends on the duration, temperature, pH and relative concentration of the reagents; the sera should be transparent and should not contain sediment; haemolyzed and contaminated sera give a non-specific reaction; insufficient washing of the virus-laden bacteria inhibits agglutination on account of the presence of unadsorbed virus;

the use of cold solutions of salt causes elution of the virus and lowers the quality of the antigen.

To obtain the virus-bacteria complex, which plays the part of the antigen in the ABV reaction, it is necessary to have a purified suspension of the virus in physiological saline andan inactivated suspension of a day-old bacterial culture (according to the standard of 2 milliard cells in 1 ml).

The inactivated bacterial suspension is prepared in the following manner. A day-old bacterial culture on MPA is flooded with 0.65% physiological saline, a suspension is made by rotating the test tube, this is decanted into a second test tube and placed into an ultrathermostat (TS-15M). From the time that a temperature of 56° is attained the suspension is warmed at this temperature for 30 - 45 minutes. Then it is diluted, according to the standard, to 2 milliard bacterial cells in 1 ml.

The virus suspension in physiological saline is mixed with the diluted inactivated bacterial suspension in equal volumes and is vigorously shaken at room temperature for 10-15 minutes, after which the obtained mixture is placed in a refrigerator at a temperature of 2-3° for adsorption. After 16-18 hours (on the following day) the obtained virus-bacteria complex is washed three times in physiological saline by centrifugation at 3000 r.p.m. for 20 minutes each time. This is necessary, since the tissue plasma, remaining in the virus suspension, possesses inhibiting properties to suppress the agglutinins. The prepared suspension of the virus-laden bacteria is used, on the same day, as the antigen in the ABV reaction.

97

We will present an example from our experience. Three carp were taken: two diseased with infectious dropsy (K and K^1) and one healthy (N) carp from another fish farm that was free of infectious dropsy. By the above described method there were prepared the antigen complexes: 2 from the skin lesions containing the virus and bacteria (K + b) and (K^1 + b), and one from the normal skin of a healthy carp without virus and with bacteria (N + b). Antisera were obtained from the carp K and K^1 that were diseased with infectious dropsy. The results of the agglutination reaction are presented in Table 8.

Table 7.

Table for calculating the results of the ABV reaction in numerical terms

Таблица 7

Таблица учета результатов реакции АБВ в цифровом выражении

A Разведение антисыворотки	B Степень интенсивности агглютинации			
	+	++	+++	++++
1:10	1	2	3	4
1:20	2	4	6	8
1:40	4	8	12	16
1:80	8	16	24	32
1:160	16	21	48	64
1:320	32	64	96	128
1:640	64	128	192	256

A. - Dilution of antiserum

B. - Degree of intensity of agglutination

Table 8.

Results of the ABV reaction with the sera of diseased carp

Таблица 8

Результаты реакции АБВ с сыворотками больных карпов

A Антигены	B Антисыворотка К					C Антисыворотка К¹				D Контроль
	10	20	40	80	160	10	20	40	80	
a N+б	—	—	—	—	—	—	—	—	—	—
b К+б	++	+	+	—	—	+++	+++	++	++	—
c К¹+б	+++	++	+	+	—	++	+	+	—	—
d б	+	—	—	—	—	—	—	—	—	—

A. - Antigens
B. - Antiserum K
C. - Antiserum K^1
D. - Control

a - N + b
b - K + b
c - K^1 + b
d - b

The numerical expression gives the following characterization of the results of the agglutination reaction that was set up:

Antigen + antiserum

(N + b)	+	K	= 0
(N + b)	+	K^1	= 0
(K + b)	+	K	= 8
(K + b)	+	K^1	= 33
(K^1 + b)	+	K	= 19
(K^1 + b)	+	K^1	= 8
b	+	K	= 1
b	+	K^1	= 0
Control			= 0

Consequently, the antigen of normal skin without virus gave a negative reactiom, while the antigens of the material from the infected skin, containing virus, gave a positive

9

agglutination reaction. The suspensions of inactivated bacteria, not loaded with virus, practically did not agglutinate when taken as the antigen, if one takes into account that small dilutions of antiserum generally give a non-specific reaction.

Not only the selection of agglutinable bacterial strains is of decisive significance in setting up the ABV reaction. The reaction proceeds in a better manner, if for the adsorption there is taken a day-old culture of bacteria on agar, which has been inactivated on the day that it is used. The adsorbing property of killed bacteria changes from the time of their storage, and with such preservatives as 0.4% formalin and 0.5% phenol it disappears completely; this, probably, is connected with a change in the sign of the electric charge, since the staining properties of bacterial cells that are subjected to physical and chemical treatment change.

We carried out studies to elucidate the possibility of utilizing for the ABV reaction not only fresh but also preserved (frozen and glycerine-treated) pathological material. The tests showed that the ABV reaction proceeds better with fresh material, somewhat worse with frozen and still worse with glycerine-treated material. Further studies should clarify the possibilities of preserving pathological material for the purpose of using this for serological reactions.

For the widespread application of a given serodiagnostic reaction it is necessary to standardize both the preparation of the titrated immune sera as well as the production of the titrated antigens, so as to subsequently obtain

comparable materials for studying the immuno-biological reactivity of fishes, the immunogenic properties of a virus and for other serological studies. In particular, attention should be paid to maintaining standard conditions for the adsorption of the virus onto the bacteria in carrying out the reaction of the agglutination of bacteria that are loaded with virus.

Our investigations on the identification of infectious dropsy of various species of fresh-water and marine fishes by means of the ABV reaction, in which there were employed as controls normal sera, normal tissue and suspensions of bacteria that were not loaded with virus, indicate the specificity of the reaction. Normal sera do not agglutinate the complex antigen and, if the ABV reaction happens to be positive, the titer of the agglutination does not exceed 1 : 40, while the titer of the serum of diseased or previously diseased and now recovered fish may be as high as 1 : 640. The ABV reaction with the complex antigen from normal tissue is always negative. The ABV reaction with a bacterial suspension without virus is also negative or the agglutination index is considerably lower than the agglutination index of a suspension of bacteria loaded with virus. In this way, from a known atiserum one may establish the presence of the virus in the infectious material or, conversely, from the known viral suspension one may establish the presence of homologous antiviral antibodies in the serum of the fishes, though for this, as has been mentioned above, it is important to carry out the ABV reaction in standard conditions.

The haemagglutination reaction has also been employed for infectious dropsy of carp. The essence of this reaction consists of the fact that the virus, which is adsorbed onto erythrocytes, agglutinates the latter, but in the presence of the homologous antiserum the haemagglutination is retarded or inhibited.

The haemagglutination reaction is set up in bacteriological test tubes with a rounded bottom, into which are introduced 0.5 or 1.0 ml aliquots of the viral material in dilutions of 1 : 10, 1 : 20, 1 : 40, 1 : 80 etc. Into the test tubes with the diluted virus is added an equal volume of a 0.5 - 2% suspension of chicken erythrocytes. As a control for spontaneous agglutination, the erythrocytes are added to physiological saline in the same volume. After mixing of the erythrocytes with the virus, the test tubes are shaken up at room temperature. After 30 - 90 minutes the results are read off by the form of the deposit on the bottom of the test tube: a stellate deposit indicates a positive reaction, a deposit in the form of an even disc indicates a negative reaction.

In this fashion may be determined the presence of virus in the pathological material. If one has a known titrated virus and the serum is taken from a fish with an unknown disease, then a diagnosis may be established by the reaction of the delay of haemagglutination. In the latter case the test serum is diluted, in the ratios of 1 : 10, 1 : 20, 1 : 40 etc., in 0.5 ml aliquots and to each dilution is added an equal volume of the known viral suspension of a fourfold titer. After a thorough mixing, to the mixture is added 1 ml of a

1 - 2% suspension of chicken erythrocytes. Serving as the control is a suspension of erythrocytes in a mixture of the virus and physiological saline, instead of serum. The titer of the serum is considered as being the highest dilution of this at which there is still no agglutination of the erythrocytes.

Obtaining chicken erythrocytes. A 1 - 2% emulsion of chicken erythrocytes in physiological saline is generally used for the haemagglutination reaction.

If a rooster-donor is present in the laboratory, the blood may be obtained once a week from the subalar vein by Erdman's procedure. The rooster is tied down to the bench by the legs and wings, not too tightly, and a sterile towel is placed under the wing. The body of the bird is also covered with a sterile towel and gauze, leaving open the site of the operation. The feathers on the site of the operation should not be pulled out but clipped. The skin is washed with sterile physiological saline. After this, Erdman recommends that a general narcosis (chloroform) be given, but there is no great need of this. The skin is wiped with a cotton wool pad, moistened with alcohol, then it is lifted up with forceps and the cut is made with sharp-tipped scissors. The edges of the cut in the skin are grasped with Péan clamps and the musculature and vein are uncovered. The vein is lifted up and clamped with an arterial clamp. A hypodermic needle, cooled on ice, is introduced into the dilated vein and at the same moment the clamp is removed. The blood flows rapidly from the hypodermic needle into previously prepared, cooled centrifuge

tubes with 1 ml of a 3.8% solution of sodium citrate. 5 ml of blood is poured into each of the centrifuge tubes. The obtained citrated blood is centrifuged at 2000 r.p.m. for 10 minutes. The sedimented erythrocytes are washed three times in a 0.85% physiological saline (for warm-blooded animals) by centrifugation. From the washed erythrocytes there is prepared a 0.5 - 2% emulsion in physiological saline, which is stored in a refrigerator for 5 days.

The complement fixation reaction* has not been applied by anyone up to the present time for viral (and bacterial) diseases of fishes. It is based on the reactions of two systems: the specific system, composed of antigen and complement, and the indicator (haemolytic) system, composed of erythrocytes and haemolytic serum. From the latter is determined the presence or absence of complement fixation in the specific antigen-antibody complex with the addition of erythrocytes and haemolytic serum.

In the case of the formation of an antigen-antibody complex, in the presence of an affinity between the antigen and the specific antibodies, the fixation of complement occurs; however in the case of an absence of a specific affinity between the antigen and antibody, the antigen-antibody complex is not formed and complement is not fixed. With the addition of erythrocytes and haemolytic serum, haemolysis occurs in the case when there is free unbound complement in the system, and, conversely, if the complement is bound to the antigen-antibody complex, haemolysis does not occur. Thus, the occurrence of haemolysis serves as the indicator of a negative

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* Presented according to A. K. Shublandze and S. A. Gaidamovich (1954).

reaction and, conversely, the absence of haemolysis indicates a positive reaction.

For the complement fixation reaction are required: antigen, antiserum (immune or serum from diseased and previously diseased and now recovered animals), complement (guinea pig serum), sheep erythrocytes, haemolytic serum.

As there have not yet been any experiments on the application of the complement fixation reaction in ichthyology, we cannot recommend any particular method of preparing the antigen for this purpose, from among the numerous existing methods employed in virology. Guided by general principles, however, in the first place there should be tested antigens prepared by the method of repeated freezing and thawing out of the infected tissue. For example, in the case of the chronic form of infectious dropsy of fishes the skin tissue in the stage of haemorrhagic swelling may be recommended for the preparation of antigen.

Preparation of antigen. The infected tissue is thoroughly ground up, either in a porcelain mortar with sand or in a homogenizer, and there is prepared a 10% suspension in physiological saline, containing 2% inactivated normal serum. The suspension is placed for 18-20 hours in a refrigerator at a temperature of 4-6°, and is then centrifuged at 2500 r.p.m. for 30 minutes. The supernatant liquid, poured out into sterile test tubes of organic glass, is frozen at a temperature of minus 72° in a mixture of solid carbon dioxide and alcohol for 20-25 minutes and then placed in a water bath at a temperature of 37° for thawing. The freezing and thawing

is repeated 5 times. The flocculi which are formed after freezing are sedimented by centrifugation for one hour at 3000 - 3500 r.p.m. To the obtained transparent liquid there is generally added, as a preservative, phenylmercuric nitrate (1 : 40 000) or merthiolate (1 : 10 000) and this is stored in a refrigerator at a temperature of 4-6° until use.

Immune serum. It is necessary to have the necessary appropriate antibodies to obtain the complex which fixes the complement. For this purpose is used the serum of recovered (convalescent) fishes or the blood serum of fishes that were artificially immunized with known virus. In this manner, using the known immune serum one can determine, by the complement fixation reaction, the presence of the virus in the investigated infectious material and establish a diagnosis of the disease.

Since the immunization is carried out with virus which is not free of tissue elements, it should be taken into consideration that in the immune serum, in addition to the specific anti-viral antibodies, there are also present non-specific anti-tissue antibodies. It is therefore necessary to attenuate the immune serum. It is diluted twofold with 0.85% physiological saline and there is added 10% (per the amount of whole serum) of ground up native tissue mass. The obtained mixture is held for 2 hours in a thermostat at a temperature of 37°, then transferred into a refrigerator for 18 hours at a temperature of 4-6°. After this it is centrifuged at 3000 r.p.m. The supernatant liquid is separated and the anti-complementary properties of the serum are eliminated by passing

carbon dioxide gas through it for 3-5 minutes. Previously the serum is diluted tenfold with distilled water. The carbon dioxide is delivered from a Kipp's apparatus (with sodium carbonate and hydrochloric acid). The cloudiness which appears in the serum is sedimented by centrifugation. The NaCl content is brought up to 0.85% of the physiological concentration by the addition of one tenth the volume of an 8.5% solution of sodium chloride. The anticomplementary properties of the serum of fishes are eliminated on the day prior to the reaction. The treated fish serum is warmed for 20 minutes at a temperature of 50°. As a control there is also prepared, in a parallel manner, immune serum of warm-blooded animals (guinea pigs or white rats).

The haemolytic serum for the haemolytic system of the reaction as well as the dry complement may be acquired in ready-made form.

The reaction is set up (according to 10
Shublandze and Gaidamovich) either with known atigen for determining the specific antibodies, or with known immune serum for determining the presence of the virus. We will consider the first variant.

The experiment is set up in a parallel manner with the virus antigen, prepared from infected tissue, and with the control antigen, prepared from similar tissue of a healthy fish.

All of the ingredients for the experiment (physiological saline, washed erythrocytes, inactivated serum) and glassware are prepared on the day before. The experiment is started

with the preparation of a 3% suspension of erythrocytes and an equal volume of a working dilution of the haemolytic serum. If, for example, the titer of the haemolytic serum is equal to 1 : 2400 and there are required 80 ml of a working dilution of serum of 1 : 800, then 0.1 ml of the haemolytic serum is taken and diluted in 79.9 ml of physiological saline. The 3% suspension of erythrocytes is prepared in physiological saline for warm-blooded animals, as are all the dilutions of the serum, since sheep erythrocytes participate in the reaction. For this 2.4 ml of the washed erythrocytes are flooded with 77.6 ml of the physiological saline.

First stage of the reaction. For determining the working dose of complement per se it is diluted with physiological saline: initially into the test tubes is poured physiological saline, then undiluted complement into test tubes Nos. 1, 2 and 4 as shown in Table 9.

Table 9.

Scheme for diluting complement.

Таблица 9

Схема разведения комплемента

A Номер пробирки	B Искомое разведение комплемента	C Количество, мл	
		a физиологического раствора	b цельного комплемента
1	1:10	1,8	0,2
2	1:15	1,4	0,1
3	1:20	0,5	—
4	1:25	2,4	0,1
5	1:30	0,5	—
6	1:40	0,5	—
7	1:60	0,5	—
8	1:80	0,5	—

A - Number of test tube
B - Desired dilution of complement
C - Amount, ml
  a - of physiological saline
  b - of undiluted complement

Table 10.

Scheme for titration of complement.

Таблица 10

Схема титрования комплемента

A Разведение комплемента	B Ингредиенты, мл			C Результат реакции
	a комплемент	b физиологический раствор	c гемолитическая система	
1:10	0,2	0,4	0,4	0
1:15	0,2	0,4	0,4	0
1:20	0,2	0,4	0,4	0
1:25	0,2	0,4	0,4	0
1:30	0,2	0,4	0,4	0
1:40	0,2	0,4	0,4	+
1:60	0,2	0,4	0,4	++

A. - Dilution of complement

B. - Ingredients, ml

a - complement
b - physiological saline
c - haemolytic system

C. - Result of reaction

Into test tubes Nos. 3, 5, 6, 7 and 8 the complement is added with a different pipette in the following manner: from the 1 : 10 dilution in test tube No. 1 is taken 0.5 ml and transferred into test tube No. 3; from the 1 : 15 dilution in test tube No. 2 is taken 0.5 ml and transferred into test tube No. 5 and so on. After the dilution of the complement one proceeds to the titration of this in the haemolytic system, which is prepared 15 minutes before use. 10

Titration proceeds according to the scheme shown in Table 10 (according to a selected example).

Into the main series of test tubes there are poured, using a separate pipette, 0.2 ml aliquots of the prepared dilutions of complement, to each test tube is added 0.4 ml of

physiological saline and 0.4 ml of the haemolytic system. The obtained mixtures of various dilution are shaken up, placed for 30 minutes into a thermostat and after 15 minutes again shaken. Complete haemolysis is indicated by a zero, absence of haemolysis - by pluses. The working dose of complement is considered to be the dilution which corresponds with the dilution in the test tube that is located one tube to the left of the test tube with complete haemolysis. In the given example this corresponds to a dilution of 1 : 30, and consequently the working dose of complement is 1 : 20.

Second stage of the reaction is the titration of the complement in the presence of antigen or serum to determine to what extent the activity of the complement is suppressed in the fixation reaction. For this purpose there are assayed still finer fractional dilutions of complement within the limits of its working dose. Complement at a dilution of 1 : 20 is poured out into 8 test ubes in a tenfold quantity (Table 11).

Table 11.

Scheme for secondary dilution of complement.

Таблица 11

Схема вспомогательного разведения комплемента

A Номер пробирки	B Искомая доза комплемента, мл	C Количество, мл a комплемента в разведении 1:20	 b физиологического раствора
1	0,04	0,4	1,6
2	0,06	0,6	1,4
3	0,08	0,8	1,2
4	0,10	1,0	1,0
5	0,12	1,2	0,8
6	0,14	1,4	0,6
7	0,16	1,6	0,4
8	0,18	1,8	0,2

A - Number of test tube
B - Desired dose of complement, ml
C - Amount, ml
    a - of complement at a dilution of 1 : 20
    b - of physiological saline.

Table 12.

Scheme for titration of complement in the presence of the basic dilution of the antigen or serum

Таблица 12

Схема титрования комплемента в присутствии основного разведения антигена или сыворотки

A Номер пробирки	B Количество, мл		
	a антигена или сыворотки	b физиологического раствора	c комплемента в соответствующих дозах
1	0,2	0,2	0,2
2	0,2	0,2	0,2
3	0,2	0,2	0,2
4	0,2	0,2	0,2
5	0,2	0,2	0,2
6	0,2	0,2	0,2
7	0,2	0,2	0,2
8	0,2	0,2	0,2

A. - Number of test tube

B. - Amount, ml
a - antigen or serum
b - physiological saline
c - complement in corresponding doses

From the obtained secondary series of test tubes the diluted complement is transferred in 0.2 ml aliquots into the test tubes with the antigen or serum according to the following scheme for titration (Table 12).

The test tubes with the various dilutions of the working dose of complement in the presence of antigen or serum are held for 18 hours at a temperature of 4°, after which there are added to them 0.4 ml aliquots of the haemolytic system. The reaction is read after 45 minutes. The recorded entry for the reaction, in accordance with the given example is presented in Table 13.

Table 13.

Record of the results of the titration of complement in the presence of ingredients.

Таблица 13

Протокол результатов титрования комплемента в присутствии ингредиентов

A Доза комплемента, мл	B Антиген		C Сыворотка реконвалесцента
	a специфический	b контрольный	
0,04	++++	++++	++++
0,06	++++	++++	++++
0,08	++++	++++	++++
0,10	++++	++++	++
0,12	++++	+++	+
0,14	++	+	0
0,16	0	0	0

A. - Dose of complement, ml

B. - Antigen
  a - specific
  b - control

C. - Serum of convalescent

Thus, the activity of the complement was expressed by the appearance of haemolysis in the presence of the antigen at a dose of 0.16 ml, and in the presence of serum - at a dose of 0.14 ml of complement at a dilution of 1 : 20. There is taken one and a half of the haemolytic unit, $\frac{0.16 \times 1.5}{20}$, multiplied by the number of test tubes required for the experiment. For example, 1.2 ml of the undiluted complement is required for 100 test tubes. Since the total volume of complement for 100 test tubes, with 0.2 ml aliquots, is 20 ml, then 18.8 ml of physiological saline are added to 1.2 ml of the undiluted complement.

The main experiment of the complement fixation reaction in this case is set up according to the scheme shown in Table 14.

The test tubes are set out in 5 series corresponding to the dilutions of the convalescent serum (immune serum) and to the control of the antigen for haemotoxicity and anticomplementary properties. 6 test tubes are set up in each series. The reaction ingredients, all in 0.2 ml aliquots, are combined in the following order: serum, antigen, complement.

Into all 6 test tubes of the first four series there is poured serum in the appropriate dilutions, then into test tubes Nos. 1 and 2 of each of these four series - the specific antigen, while into test tubes Nos. 3 and 4 of these same series - the normal tissue antigen. Test tubes Nos. 5 and 6 serve as a control for anticomplementarity. In the fifth series test tubes Nos. 1, 2, 4 and 5 serve as a control of the antigens without serum, while test tubes Nos. 3 and 6 serve as a control of the antigens for haemotoxicity.

Then complement is added: to the odd-numbered test tubes of each series - 1½ doses of complement in each, while to the even-numbered test tubes - 2 doses in each. Complement is not added to test tubes Nos. 3 and 6 of the fifth series.

First phase of reaction: the test tubes with the ingredients, mixed together, as shown in the scheme, are placed in a refrigerator at a temperature of 4° for 18 - 20 hours.

Second phase of reaction: after 18 - 20 hours the test tubes are removed from the refrigerator, to them is added

Table 14.

Scheme for setting up the main experiment.

Таблица 14

Схема постановки главного опыта

A	B	C	D Количество, мл					
Номер ряда	Сыворотка реконвалесцента в разведении	Номер пробирки	a специфического антигена	b антигена нормальной ткани	c комплемента 1,5 дозы	d комплемента 2 дозы	e физиологического раствора	f гемолитической системы при температуре 37°
1	1 1:5 (во все пробирки по 0,2 мл)	1	0,2	—	0,2	—	—	0,4
		2	0,2	—	—	0,2	—	0,4
		3	—	0,2	0,2	—	—	0,4
		4	—	0,2	—	0,2	—	0,4
		5	—	—	0,2	—	0,2	0,4
		6	—	—	—	0,2	0,2	0,4
2	2 1:10 (во все пробирки по 0,2 мл)	1	0,2	—	0,2	—	—	0,4
		2	0,2	—	—	0,2	—	0,4
		3	—	0,2	0,2	—	—	0,4
		4	—	0,2	—	0,2	—	0,4
		5	—	—	0,2	—	0,2	0,4
		6	—	—	—	0,2	0,2	0,4
3	3 1:20 (во все пробирки по 0,2 мл)	1	0,2	—	0,2	—	—	0,4
		2	0,2	—	—	0,2	—	0,4
		3	—	0,2	0,2	—	—	0,4
		4	—	0,2	—	0,2	—	0,4
		5	—	—	0,2	—	0,2	0,4
		6	—	—	—	0,2	0,2	0,4
4	4 1:40 (во все пробирки по 0,2 мл)	1	0,2	—	0,2	—	—	0,4
		2	0,2	—	—	0,2	—	0,4
		3	—	0,2	0,2	—	—	0,4
		4	—	0,2	—	0,2	—	0,4
		5	—	—	0,2	—	0,2	0,4
		6	—	—	0,2	0,2	0,2	0,4
5	5 Контроль антигена на гемотоксичность и антикомплементарные свойства	1	0,2	—	0,2	—	0,2	0,4
		2	0,2	—	—	0,2	0,2	0,4
		3	0,2	—	—	—	0,4	0,4
		4	—	0,2	0,2	0,2	0,2	0,4
		5	—	0,2	—	0,2	0,2	0,4
		6	—	0,2	—	—	0,4	0,4

A - Number of series
B - Convalescent serum diluted
C - Number of test tube
D - Amount, ml
  a - of specific antigen
  b - of normal tissue antigen
  c - of complement, 1½ doses
  d - of complement, 2 doses
  e - of physiological saline
  f - of haemolytic system at a temperature of 37°

1 - 1:5 (0.2 ml in all test tubes)
2 - 1:10 (")
3 - 1:20 (")
4 - 1:40 (")
5 - Control of antigen for haemotoxicity and anticomplementary properties

Table 15.

Form of record of the main experiment of the complement fixation reaction.

Таблица 15

Форма протоколов главного опыта реакции связывания комплемента

A Название сывороток и контроля антигена	B Разведение сыворотки	C Доза комплемента a полуторная x антиген специфический	C a полуторная y антиген нормальной ткани	C a полуторная z контрольная сыворотка	C b двойная x антиген специфический	C b двойная y антиген нормальной ткани	C b двойная z контрольная сыворотка
1. Сыворотка реконвалесцента	1:5	++++	0	0	++++	0	0
	1:10	++++	0	0	++++	0	0
	1:20	++++	0	0	+++	0	0
	1:40	++++	0	0	++	0	0
2. Сыворотка нормальная	1:5	0	0	0	0	0	0
	1:10	0	0	0	0	0	0
	1:20	0	0	0	0	0	0
	1:40	0	0	0	0	0	0
3. Контроль антигена на антикомплементарные свойства		0	0	0	0	0	0
4. Контроль антигена на гемотоксичность		++++	++++				

A - Designation of sera and antigen controls

B - Serum dilution

C - Dose of complement

a - one and a half
b - double

x - specific antigen
y - normal tissue antigen
z - control serum

1. - Convalescent serum

2. - Normal serum

3. - Control of antigen for anticomplementary properties

4. - Control of antigen for haemotoxicity

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the haemolytic system and they are placed in a thermostat at a temperature of 37° for 30-45 minutes. In the test tubes in which the complement is fixed by the specific complex the haemolysis will be inhibited, while in the test tubes in which the complement remains free complete haemolysis occurs.

The record entry is made according to the form shown in Table 15.

In the given example the specific antibodies were detected in the convalescent serum at a titer of 1 : 40, i.e. the antigen-antibody complex, which was formed, fixed the complement at a serum dilution of 1 : 40.

If the presence of virus in the infectious material is being determined by the complement fixation reaction from the known immune serum (or convalescent serum), the experiment is set up according to the same scheme, but different dilutions of antigen are tested with a constant dose of serum.

EQUIPMENT OF VIROLOGICAL AND BACTERIOLOGICAL LABORATORIES FOR THE STUDY OF INFECTIOUS DISEASES OF FISHES

The equipment in virological and bacteriological laboratories is distributed in accordance with the technique of conducting the studies. In the laboratory there should be: an auxiliary room; a preparation room (for washing glassware, preparing the nutrient media, sterilization); aquarium and vivarium rooms; a room for working with the cultures (with cubicles for transplantations of the bacterial cultures and tissue cultures); a room for microscopic study of the preparations.

The auxiliary room is to be used for the storage of various laboratory materials and feed for the fish, rabbits and other animals used in the experiments, for the storage of field equipment etc. Next to this should be a room for the preparation of animals for the experiments and for pathological dissections. On one side of the auxiliary room it is convenient to locate the aquarium and vivarium rooms, on the other side - the preparation room.

The aquarium room. In this are located aquaria of various dimensions, in the construction of which it is necessary to take into consideration the water flow, aeration, temperature, artificial daylight and feeding. Depending on the subject and aim of the experiment all of these conditions may be set up in the most varied combinations. For this every aquarium is furnished with an independent water supply with a separate cock to regulate the water exchange.

The required level of water is maintained by means of an extensible drain tube, which is inserted into the bottom of the aquarium into a screw coupling with a rubber gasket on the opposite side with respect to the water inflow cock. Onto the drain tube is placed a hollow cylinder with slits in its wall at the bottom for circulation of the bottom layer of water and cleansing of the aquarium. The slits in the wall at the bottom of the cylinder should be no wider than 3-4 mm (thickness of fish body); their length - 40-50 mm. 111

Into each aquarium is supplied air from an air pump or oxygen from a compressed gas cylinder. Onto the gas outlets are mounted lengths of thin rubber tubing with with gas dispersal devices which are placed on the bottom of the aquarium in such a way that the water becomes evenly saturated with oxygen. The content of the dissolved oxygen in the water is generally determined by titration according to Winkler's method.

For maintaining the water temperature, necessary for a given experiment, into each aquarium there is placed a separate electric heater with a thermoregulator for obtaining temperatures of 15, 20 and 25°, or there is fed in hot water which is diluted through a mixer with cold water.

The aquaria should be evenly illuminated by daylight lamps. Mounting these lamps directly on the aquaria is undesirable, since the fish become frightened when the lights are switched on. Beside each aquarium are placed additional normal electric lamps, which are switched on with a gradual increase of the incandescence.

The food used depends on the species of experimental fish, with preference given to natural foods (mosquito larvae, worms etc.). It is easy to train carp, crucian carp and orfe to artificial food under aquarium conditions. During feeding it is necessary that the remaining unconsumed food is removed at the proper time, since the food remains are a favourable medium for the development of the Saprolegnia fungus, the hyphae and spores of which cause tissue necrosis when they fall onto the traumatized surface of the body of fishes. Fish often die of Saprolegnia, a dangerous enemy of aquarium fishes. A solution of methylene blue (1 : 50 000 or 1 : 100 000) is employed successfully for controlling Saprolegnia. A. I. Kanaev recommends liquid chlorine as a universal disinfectant. The water is chlorinated with the help of a special dosimetric installation.

The preparation room. Here the main preparation work is carried out. In the room there should be a wash sink with running hot and cold water for washing the laboratory glassware. For the drying and sterilization of the glassware, and for the preparation and sterilization of the artificial media it is necessary to have electrical equipment: drier to 200-250°; autoclave; Koch apparatus; hot-plate (if there is no gas); centrifuge to 5000-6000 r.p.m.; refrigerated ultracentrifuge to 30 000 r.p.m.; still for obtaining distilled water; electrical or water-jet vacuum pump for filtration and drying; agitator (mechanical shaker); lamp potentiometer.

A chemical fume cupboard is located in the preparation room for work with acids.

The room for working with cultures should be well ventilated, the floor should be covered with linoleum ("relin"*), while the walls and ceiling are painted with an oil point. A part of the room is allocated to cubicles with an antecubicle for the isolation of pure bacterial cultures and for transplantation, and a separate cubicle for work with tissue cultures. The cubicle space is sterilized by means of BUV-15 (or BUV-30) bacteriocidal lamps. In each cubicle there should be no less than two work areas with an adequate air cubic capacity (3 X 4 X 4). Bacteriocidal lamps are mounted on the ceilings within the cubicles and antecubicle. The work areas are illuminated by a reflector from the outside through a glass partition.

The sterile coats, gauze masks, caps and other gear for working in the cubicle is kept in the antecubicle. At the entrance to the antecubicle on the floor there is placed a foam rubber mat impregnated with antiseptic (a 5% lysol solution, 1% solution of chloramine).

For supplying sterile air into the cubicle for tissue cultures the Moscow Research Institute for Viral Preparations has elaborated an installation composed of: filters for the coarse purification of air by the Rekk** system; a Sirokko** system ventilator; an air cooler in the form of a hermetic housing containing evaporators cooled by a freon refrigeration unit; sterilizing filters with FPP-15-3 filtering material.

The routine bacteriological work is conducted outside the cubicles in this room.

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* Translator's note. "relin" - direct transliteration. Pbly a trade name.

** Translator's note. Direct transliterations (Reck?, Sirocco?).

Basic equipment for work with cultures: polythermostat or separate thermostats set at temperatures of 20, 25, 30 and 37°; refrigerator of the ZIL type; homogenizer (tissue microtriturator); magnetic stirrer; refrigerated centrifuge to 10 000 r.p.m.; water jet pump.

The microscopy room should be separate from the room used for the culture work, since with inadequate ventilation in the latter there accumulate smells, which are harmful to human health, from the products of the vital activity of the bacterial cultures (mercaptan, methane et al.). For the normal microscopy of fine cytological structures the work areas are equipped with an artificial light source, employing our domestically produced OI-19 illuminator. For luminescent microscopy there is utilized the normal MBI-3 microscope with an OI-18 illuminator and an SVD-120A mercury lamp, an electical control panel and set of light filters. However it is better to have the ML-2 luminescent microscope. The luminescent microscopy is carried out in a special dark room with good ventilation.

APPENDICES

11

List of basic equipment for virological and bacteriological laboratories

Optics

Agglutinoscope
AU-12 binocular attachment
OI-13 dark field condenser
Magnifiers: folding, preparation, headpiece
MBS-1, MBS-2 microscope-magnifier.
MBI-1, MBI-3 microscopes, ML-2 field microscope
Stage micrometer
Eyepiece micrometer (ruled line)
OI-19 illuminator for microscope
OI-18 illuminator for luminescent microscopy and an SVD-10A mercury lamp or better a DRSh-250 with an electrical control panel and set of light filters
RDU refractometer (Kiev)
RA-4 projection apparatus
Phase-contrast equipment
MFN-1 photo-attachment
FEKN-57 photoelectric colorimeter

Equipment

A-25, AE-3, AV-2 autoclaves
Ullengut filtration apparatus
Water bath
Carbon dioxide cylinder
Technical balance to 1 kg with set of weights
Damped balance
Torsion balance to 500 mg
Centrifuge balance
Still

Surgical instruments (scissors, scalpels, knives, forceps, Péan clamps etc.)
Flask heater
Goryaev chamber
Chamber for paper (or gel) electrophoresis with rectifier and densitometer
Enamel trays
Micromanipulator
Sliding block microtome
Freezing microtome
Micro-triturator for tissues (homogenizer)
VN-461 vacuum oil pump
Komovskii pump
Electric hot-plate
LPU-01 potentiometer for pH determinations
Fine-pored candles for filtration
Sterilizer
Drying cabinet
S-450, TS-15-M thermostats
Thermos flasks for food
ZIL-Moskva refrigerator
Refrigerated centrifuges for 3000 and 10 000 r.p.m.
Hand centrifuge
1, 5 and 10 ml Rekord syringes with needles

Laboratory ware

Large bottles (of various sizes)
Enamel pans
Bunsen flasks with tubulure
Measuring flasks
Erlenmeyer flasks of various volumes, narrow-necked
Narrow-necked flat-bottomed flasks of various volumes
Crystallizing dishes
Measuring pipettes of various volumes
Cover slips
Microscope slides (thin)

Centrifuge tubes, simple and calibrated test tubes
Bacteriological test tubes
Serological test tubes of 10 mm diameter
Serological test tubes of 8 mm diameter
Glass funnels
Porcelain mortars
Pipette cleaning wires
Measuring cylinders of various volumes
Watch glasses
Porcelain dishes
Porcelain Guch funnels

Form for recording observations

No . . . . . Date . . . . . .

Name of body of water

Pollution . . . . . .
Development of vegetation . . . . . .
Degree of silting . . . . . .
Bloom . . . . . .
Other characteristics . . . . . .

Name of species of subject

Sex . . . . . Weight . . . . . Size . . . . . Age . . .

Symptoms of disease

Type of behaviour, of swimming . . . . . .
Colour of skin . . . . . .
Local lack of pigmentation (patches) . . . . . .
Dilatation of blood vessels and capillaries . . . . . .
Haemorrhages (punctate, diffuse) . . . . . .
Type of ulceration . . . . . .
Wounds on skin . . . . . .
Thickenings (infiltrates) . . . . . .
Abscesses . . . . . .
Mortifications (necroses) . . . . . .

General oedema . . . . . .
Local oedema . . . . . .
Swellings on body, head, jaws, fins . . . . . .
Ruffling of scales . . . . . .
Bulging of eyes . . . . . .
Inflammation of anus . . . . . .
Colour of gill filaments . . . . . .
Presence of ectoparasites . . . . . .
Presence of Saprolegnia fungus . . . . . .

Data from pathologico-anatomical dissections

Abdominal cavity . . . . . .
Intestine . . . . . .
Liver . . . . . .
Gall bladder . . . . . .
Bile . . . . . .
Kidney . . . . . .
Gonads . . . . . .
Heart . . . . . .
Pericardial fluid . . . . . .
Brain . . . . . .
Swim bladder . . . . . .

Form of record card for bacterial cultures

Institution . . . . . .

No . . . . . Date . . . . . Body of water . . . . . .

Colony in surface view . . . . . .
Subject of study . . . . . .
Features of disease . . . . . .
Pathologico-anatomical changes . . . . . .
Isolated from . . . . . Colony in profile . . . .
Temperature optimum . . . . . .
Morphology . . . . . .

Motility	
Flagella	
Spore formation	
Capsule formation	
Gram staining	
Growth on bouillon	
Growth on agar	
Formation of pigment	
Growth on gelatin	
Growth on litmus milk	
Growth on potato	
Fermentation of glucose	
Fermentation of lactose	
Fermentation of mannitol	
Fermentation of saccharose	11
Fermentation of maltose	
Relation to oxygen	
Hydrogen sulphide	
Ammonia	
Reduction of nitrates	
Indol	
Neutral red	
Methyl red	
Acetyl methyl carbinol	
Fat	
Starch	

BIBLIOGRAPHY 113

1. Goncharov G. D. Elementary bodies of the virus of fish pox. - "Voprosy virusologii" ("Problems of virology"), 1960, No. 2.

2. Goncharov G. D. Immunological reactivity in fishes. - Byulleten' IBVV AN SSSR (Bulletin of the Institute for the Biology of Inland Waters of the USSR Academy of Sciences), 1962, No. 2.

3. Goncharov G. D. Immunity in fishes. - Trudy 4-go Vsesoyuznogo soveshchaniya po boleznyam ryb (Transactions of the 4th All-Union Conference on fish diseases), 1964.

4. Goncharov G. D., Zubkova L. A. and Stepanova L. A. Monolayer cell culture of kidney tissue of bream. - Informatsionnyi byulleten' "Biologiya vnutrennikh vod" AN SSSR, (Informational bulletin "Biology of Inland Waters" USSR Academy of Sciences), 1970, No. 6.

5. Dolinov K. E. Fundamentals of the technology of dry live vaccines. - In the book: Prophylaxis of infections by live vaccines. Moscow, Medgiz (Medical Publishers), 1960.

6. Zil'ber L. A. The study of viruses. Moscow, Medgiz, 1956.

7. Kalina G. P. Ultrafiltration in microbiology, methods and apparatus. - In the book: Textbook on the microbiology, clinical aspects and epidemiology of infectious diseases. Moscow, Medgiz, 1962

8. Kolesov S. G. Desiccation of microorganisms and biological preparations. Moscow, 1952.

9. Tissue culture in virological studies. - Authors: - O. G. Andzhaparidze, V. I. Gavrilov, B. F. Semenov, S. G. Semenova. - Moscow, Medgiz, 1962.

10. Mikryakov V. R. Detection of specific immunoglobulins in fishes with the aid of ascending paper chromatography by Hastaned's method. - Informatsionnyi byulleten' Instituta biologii vnutrennikh vod AN SSSR (Informational bulletin of the Institute for the Biology of Inland Waters of the USSR Academy of Sciences), 1970, No. 7.

11. Usadchaya E. F. The isolation of cytopathogenic agents from carp in the acute form of infectious dropsy. - "Veterinariya" ("Veterinary Medicine"), 1964, No. 2.

12. Usadchaya E. F. Utilization of primary monolayer cell cultures of carp for the isolation of the cytopathogenic agent from fishes with infectious dropsy disease. - "Rybnoe khozyaistvo" ("The fishing industry"), 1970, No. 10.

13. Peshkov M. A. The cytology of bacteria. Publishing House of the USSR Academy of Sciences, 1955.

14. Peshkov M. A. The light microscope and its variants. - In the book: Textbook on the microbiology, clinical aspects and epidemiology of infectious diseases. Moscow, 1962, vol. 2.

15. Predtechenskii V. E. Textbook on clinical laboratory studies. Moscow, Medgiz, 1960.

16. Revo M. V. and Zhukova N. D. Veterinary microbiology. - Moscow, 1958.

17. Romeis B. Microscopic technique (translated from the German). Moscow, 1953.

18. Syurin V. N. Methods for virological studies. - In the book: Veterinary laboratory practice. Moscow, 1963, vol. 1.

19. Shubladze A. K. and Gaidamovich S. Ya. A short course in practical virology. Moscow, 1964.

22. Dubowicz M. and Kocylowski B. Studies on the presence of a viltrable virus in cases of infectious dropsy of carp. Roczniki Nauk Rolniczych (Annals of Agricultural Sciences), Series E., vol. 67, No. 4, pp. 517-527.

26. Grützner L. - Investigation of some practical possibilities of tissue culture s of Lebistes reticulatus (Peters) and Lacropodus opercularis (Linné) in virus research.

27. Grützner L. In vitro rearing of liver and kidney tissue of Tinca vulgaris Cuv. (Schleie) in trypsinized monolayer tissue cultures

УКАЗАТЕЛЬ ЛИТЕРАТУРЫ

1. Гончаров Г. Д. Элементарные тельца вируса оспы рыб. — «Вопросы вирусологии», 1960, № 2.
2. Гончаров Г. Д. Иммунологическая реактивность у рыб. — Бюллетень НИВВ АН СССР, 1962, № 2.
3. Гончаров Г. Д. Иммунитет у рыб. — Труды 4-го Всесоюзного совещания по болезням рыб, 1964.
4. Гончаров Г. Д., Зубкова Л. А., Степанова М. А. Однослойная клеточная культура почечной ткани леща. — Информационный бюллетень «Биология внутренних вод» АН СССР, 1970, № 6.
5. Долинов К. Е. Основы технологии сухих живых вакцин. — В кн.: Профилактика инфекций живыми вакцинами. М., Медгиз, 1950.
6. Зильбер Л. А. Учение о вирусах. М., Медгиз, 1956.
7. Калина Г. П. Ультрафильтрация в микробиологии, методы и аппаратура. — В кн.: Руководство по микробиологии, клинике и эпидемиологии инфекционных болезней. М., Медгиз, 1962.
8. Колесов С. Г. Высушивание микроорганизмов и биопрепаратов. М., 1952.
9. Культура ткани в вирусологических исследованиях. — Авт.: О. Г. Анджапаридзе, В. Н. Гаврилов, Б. Ф. Семенов, С. Г. Семенова. — М., Медгиз, 1962.
10. Микряков В. Р. Выявление специфических иммуноглобулинов у рыб с помощью восходящей хроматографии на бумаге по методу Кастанеда. — Информационный бюллетень Института биологии внутренних вод АН СССР, 1970, № 7.
11. Осадчая Е. Ф. Выделение цитопатогенных агентов от карпов при острой форме краснухи. — «Ветеринария», 1964, № 2.
12. Осадчая Е. Ф. Использование первичных однослойных клеточных культур карпа для выделения цитопатогенного агента от рыб, больных краснухой. — «Рыбное хозяйство», 1970, № 10.
13. Пешков М. А. Цитология бактерий. Издательство АН СССР, 1955.
14. Пешков М. А. Световой микроскоп и его разновидности. — В кн.: Руководство по микробиологии, клинике и эпидемиологии инфекционных болезней. М., 1962, т. 2.
15. Предтеченский В. Е. Руководство по клиническим лабораторным исследованиям. М., Медгиз, 1960.
16. Рево М. В., Жукова М. Д. Ветеринарная микробиология. М., 1958.
17. Ромейс Б. Микроскопическая техника (перевод с нем.). М., 1953.
18. Сюрин В. Н. Методы вирусологических исследований. — В кн.: Ветеринарная лабораторная практика. М., 1963, т. 1.
19. Шубладзе А. К., Гайдамович С. Я. Краткий курс практической вирусологии. М., 1954.
20. Bodian D. Simplified method of dispersion of monkey kidney cells with trypsin. — Virology, 1956, v. 2, N 4, p. 575.
21. Breed R. S., Murray E. G. D., Smith N. R., Bergey's manual of determinative bacterioligy. Fth ed. Baltimore, Williams and Wilkins, 1957, p. 1094.
22. Dubowicz M., Kocylowski B. Badania nad obecnością, zarazka przesaczalnego w przebiegu posocznicy karpi. — Roczn. Nauk roln., Ser. E., 1956, v. 67, N 4, p. 517—527.
23. Dulbecco R. Production of plaques in monolayer tissue cultures by single particles of an animal virus. — Proc. nat. Acad. Sci., 1952, v. 38, N 8, p. 747—752.
24. Dulbecco R., Vogt M. Plaque formation and isolation of pure lines with poliomyelitis viruses. — J. exp. Med., 1954, v. 99, N 2, p. 167—182.
25. Fryer J. L., Vusha A., Pilcher K. S. The in vitro cultivation of tissue and cells of pacific salmon and steelhead trout. — Ann. N. Y. Acad. Sci., 1965, v. 126, N 1, p. 566—586.
26. Grützner L. Überprüfung einiger Anwendungsmöglichkeiten der Gewebekultur von Lebistes reticulatus (Peters) und Macropodus opercularis (Linné) in der Virusforschung. — Zbl. Bakteriol. I. Abt., Orig., 1956, Bd. 165, N 2—3, s. 81—96.
27. Grützner L. In vitro — Züchtung des Leber — und Nierengewebes von Tinca vulgaris Cuv. (Schleie) in trypsinierten Einschichtgewebekulturen. — Zbl. Bakteriol. I. Abt., Orig., 1958, Bd. 173, N 3/4, s. 195—201.
28. Isolation of the viral causative agent from the ocule form of indectious dropsy of carp. — Vet. Arhiw, 1971, v. 41, N 5—6, p. 125—133.
29. Paul J. Cell and tissue culture. 3-d ed. London, Williams and Wilkins, 1965, p. 371.
30. Pfitzner I. Cell and tissue culture of fresh water fish in virus researrch. — 1965, Ann. N. Y. Acad. Sci., v 126, N 1, p. 547—554.
31. Preparation of monolager cell cultures from tissues of some lower vertebrates. — Science, 1960, v. 132, N 3443, p. 1890—1891. — Aut.: K. Wolf, M. C. Quimby, E. A. Pyle, R. P. Dexter.
32. Rappaport C. Trypsinisation of monkey kidney tissue. — Bull. World Health Org., 1956, v. 14, N 1, p. 147—166.
33. Tomasec I. I., Fijan N. N. The cytomology of infectious dropsy of carp. — Ann. N. Y. Acad. Sci. 1965, v. 126, N 1.
34. Weissenberg R. Positive results of filtration experiment suppotring the view that agent of lymphocystis. — Anat. Rec., 1951, v. 111.
35. Wolf K., Dunbar C. E. Cultivation of adult teleost tissues in vitro. — Proc. Soc. exp Biol. Med., 1957, v. 95, N 3, p. 455—458.
36. Wolf K., Quimby M. C. Established eurythermic line of fish cells in vitro. — Science, 1962, v. 135, N 3508, p. 1065—1066.
37. Youngner J. S. Monolayer tissue cultures. — I. Preparation and standardization of suspensions of trypsin— dispersed monkey kidney cells. — Proc. Soc. exp. Biol. Med., 1951, v. 85, N 2, p. 202—205.

CONTENTS

	Russian text	Translation
Authors foreword	3	3
Introduction	5	5
Methods of bacteriological investigation	7	7
Preparation and sterilization of bacteriological vessels	8	8
Preparation of basic nutrient media	9	10
Sampling pathological material for bacteriological study	19	24
Isolation of pure bacterial cultures	23	31
Cytological study of bacteria	25	34
Identification of isolated strains of bacterial cultures	33	46 *
Determination of the pathogenic properties of bacteria on fishes	41	59
Serological studies for the identification of bacteria and the diagnosis of infectious diseases	46	65
Methods of virological investigation	53	77
Collection of virological material for study	54	80
Fixation of material	59	85
Microscopy of smears, impressions and histological sections	59	86
Revealing the viral nature of fish diseases	67	97
Experimental infection of fishes and the passaging of a virus	67	99
Treatment of glassware, rubber tubing and stoppers	76	112
Preparation of nutrient media and salt solutions for tissue cultures	77	113

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* Translator's note. Owing to an error made in the translation pagination there is an extra page, 45a, between pages 45 and 46.

	Russian text	Translation
Methods of obtaining tissue cultures and infecting these with viral material	84	124
Serological methods of study	95	141
Equipment of virological and bacteriological laboratories for the study of infectious diseases of fishes	110	164
Appendices	114	169
Bibliography	118	174

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